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**INVESTIGATION OF HUMAN PAPILLOMA VIRUSES INFECTIONS
IN PROSTATE CANCER**

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SCHOOL OF SCIENCE AND TECHNOLOGY
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**A THESIS SUBMITTED TO MIDDLESEX UNIVERSITY
FOR THE TITLE OF DOCTOR OF PHILOSOPHY**

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ABBREVIATIONS

| | |
|-------|------------------------------------|
| Ad | Adenovirus |
| BPV | Bovine papillomavirus |
| BPVs | Bovine papillomaviruses |
| CR1 | Conserved region 1 |
| CR2 | Conserved region 2 |
| CRPVs | Cottontail rabbit papillomaviruses |
| CKIs | Cyclin-dependent kinase inhibitors |
| DNA | Deoxyribonucleic acid |
| DRE | Digital rectal examination |
| EGF | Epidermal growth factor |
| FAK | Focal adhesion kinase |
| E&H | Haematoxylin and Eosin |
| HIER | Heat-induced epitope retrieval |
| HDACs | Histone deacetylases |
| HPVs | Human papilloma viruses |
| LCR | Long control region |
| NCR | Non-coding region |
| NES | Nuclear export sequence |

| | |
|------|------------------------------------------|
| ORFs | Open reading frames |
| PBS | Phosphate-buffered saline |
| PDGF | Platelet-derived growth factor |
| PCR | Polymerase Chain Reaction |
| PODs | Promyelocytic oncogenic domains |
| PODs | Promyelocytic oncogenic domains |
| PSA | Prostate-specific antigen |
| pRb | Retinoblastoma tumour suppressor protein |
| RT | Room temperature |
| TCC | Transitional cell carcinoma |
| TRUS | Trans-rectal Ultrasound |
| TURP | Transurethral resection of prostate |
| UV | Ultraviolet |
| UAE | United Arab Emirates |
| URR | Upstream regulatory region |
| VLP | Virus-like particles |

DECLARATION

I declare that this thesis has been composed and written entirely by myself, and the work contained herein to have been principally conducted by myself.

Mous'ab Azzawi

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ABSTRACT

Human papillomavirus (HPV) infections are associated with benign and malignant lesions of the female and male anogenital tract. In the current study, we aimed to investigate the role of high-risk HPVs infection in the pathogenesis of prostate cancer among nations or ethnic groups, in addition to testing the role of homozygosity of arginine form at codon 72 of the *p53* gene among prostate cancer patients whose prostate tissues were infected with high-risk HPVs.

Formalin-fixed paraffin-embedded tissue samples of 123 primary prostate adenocarcinoma cases and 267 control tissues of benign prostatic hyperplasia were used in the study. Genomic DNA was purified and amplified through MY09/MY11 degenerate primers, GP5+/GP6+ consensus primers, SPF1/2 cocktail of six primers using conventional, multiplex and nested PCR techniques, and subsequently subjected to viral load quantification, genotyping, testing of polymorphism of codon 72 of the *p53* gene and apoptosis index assessment by *in situ* assay. Also, the status of the *p53* tumour suppressor gene, p16^{INK4a} transcription factor as well as the E6 protein of the high risk HPVs have been tested by immunohistochemistry in both the study and control groups.

High-risk HPVs were detected in 30 of 123 (24.3%) PCa and 16 of 267 (5.9%) BPH samples with positive HPV-DNA. The detection rate of the high-risk HPV infections was 4%, 44% and 29% among the ethnic subgroups from the Middle Eastern, Caucasian, and Afro-Caribbean of the PCa patients. There was no association between the existence of high-risk HPV infections and their viral load in PCa patients and the tumour staging, grading, PSA level and patient survival rate in those patients. Likewise, there was no significant difference in the frequency of *p53* Arg

homozygosity between the high-risk HPV-positive and the HPV-negative PCa samples. Moreover, it has been found that the existence of the high-risk HPV E6 protein within the PCa samples was independent of the status of the *p53* gene, *p16^{INK4a}* transcription factor, and the apoptosis index in these samples.

Our data showed that HPV infections do exist in PCa and BPH samples with different prevalence within ethnic groups with the least occurrence in the Middle Eastern patients. However, the infections with high-risk HPVs are not associated with the prostate cancer grade, stage, patient's PSA level, and survival rate. Therefore, our data do not support the role of HPV infection in the pathogenesis of prostate carcinoma.

CHAPTER 1: INTRODUCTION

1.1. Prostate cancer epidemiology

Prostate cancer accounts for around 13% of male deaths from cancer in the UK and it is considered the second most common cause of cancer deaths in men after lung cancer and the first most common cancer in non-smoking men. There are around 26,400 new cases per year and approximately 9,600 deaths per year in the United Kingdom [1].

Survival from prostate cancer has greatly improved since the 1970s, and rates now stand at 84%, 60%, and 28% at one, five and ten years respectively. One reason for the improved survival could be attributed to the huge increase in the number of patients presenting with early stage over the past 10 years, due to the implementation of prostate-specific antigen (PSA) testing as a core approach for urinary tract symptoms assessment in elderly men by the general practitioners. The reason may be attributed to the wide adoption of the regular digital rectal examination (DRE) of the prostate by almost all the family doctors in the United Kingdom for all adult men over 45 years old when the patients present themselves with signs and/or symptoms of a prostatic pathology [2].

The highest prostate cancer incidence rates are in the developed world and the lowest rates are in Africa and Asia [3]. There are no sufficient data about the prostate cancer epidemiology and mortality from the Middle East and Afro-Caribbean countries [4]. The extremely high rate in the USA (125 per 100,000), more than twice the reported rate in the UK (52 per 100,000), is

likely to be due to the particularly high rates of PSA testing in the USA or can be attributed to other environmental and social factors [5]. Figure 1-1 shows the incidence and mortality rates in a number of countries in the world during the year 2002 [3, 6, 7].

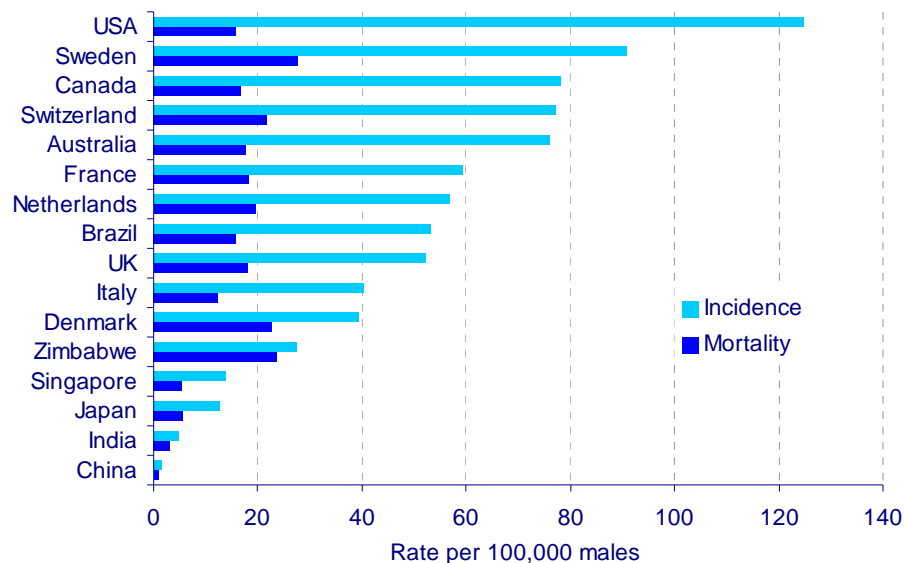


Figure 1-1: Age standardised (world) incidence and mortality rates of prostate cancer in selected countries during the year 2002.

1.2. Prostate cancer risk factors

There are several risk factors correlated with the development of prostate cancer, with different levels of involvement in the complexity of the prostate cancer pathogenesis.

Age is one of the most important factors in developing prostate cancer. The disease is very rare below the age of 40 years, but becomes increasingly common with increasing age [8]. However, most prostate cancers do not achieve clinically recognizable and aggressive states and that is why many cases of prostate cancer are only diagnosed during post-

mortem examination. However, increasing age is associated with a higher risk of high-grade prostate cancer in men of different ethnic origins [9].

The incidence of prostate cancer is 10 times higher in the western world than Asian countries [10]. This might be explained by genetic factors [11]. However, the incidence of prostate cancer also tends to increase in Asian immigrants to western countries, mainly after one generation. This may suggest the involvement of environmental factors as well [12].

Black skinned men from African black origin have the highest incidence and mortality from prostate cancer in the world. In the UK, the incidence of prostate cancer is approximately triple in men of African origin than in white men, and lowest in Asian and Middle Eastern men, suggesting a strong ethnic influence [13]. Even after adjusting for stage at diagnosis, black men have higher mortality rates than white men. Multiple reasons have been postulated to explain these findings including access to care, attitudes about care, socio-economic and education differences [14, 15], differences in type and aggressiveness of treatment, dietary, and genetic differences [11]. While each reason may contribute to the higher incidence or higher mortality, likely combination of reasons will best explain all the findings. Also, with recent advances in the understanding of genetic variations in the human genome, in general, and in the genes involved in pathways relevant to prostate cancer biology, in particular; a number of genes with alleles which differ in frequency between black and white men have been proposed as a genetic cause or contributor to the increased prostate cancer risk in black men [13].

It has been shown that the prevalence of prostate carcinoma in the UAE, like other Arabian Gulf and Asian countries, is very low compared to

Western countries despite the high intake of calories and consumption of animal fat, which are considered as contributing factors to the increased risk of development of prostate cancer [16, 17]. However, interaction of genetic and environmental factors believed to be involved in the complex aetiology of prostate cancer in those countries and elsewhere await further investigations [18-20].

It has been reported that there is a strong association between early onset of prostate cancer and the positive family history of the disease [21]. This is mainly governed by the number of relatives with prostate cancer and their age at diagnosis. Dominant pattern of inheritance with high penetrance is responsible for 5% to 10% of all prostate cancer cases, and as much as 30% to 40% of early onset disease [22].

Inflammation is frequently present in prostate biopsies, radical prostatectomy specimens and tissue resected for treatment of benign prostatic hyperplasia. Also, inflammatory infiltrates are often found in and around foci of atrophy that are characterized by an increased proliferative index. These foci, called proliferative inflammatory atrophy, may be precursors of early prostate cancer or may indicate an intra-prostatic environment favourable to cancer development. Epidemiological studies have indirectly examined the role of chronic inflammation in prostate carcinogenesis through studies of pro-inflammatory and anti-inflammatory factors. When taken together studies of sexually transmitted infections, clinical prostatitis, and genetic and circulating markers of inflammation and response to infection hint at a link between chronic intra-prostatic inflammation and prostate cancer[23]. However, there has been an increasing evidence of the association of chronic inflammation of

the prostate in the form a chronic prostatitis, which can be septic or aseptic, with the development of prostate cancer [24].

Being a hormone dependant cancer, one of the postulated theories about the pathogenesis of prostate cancer was based on the elevated androgen level in Afro-Caribbean men [25-27]. While reports of racial differences in gonadal steroid hormone levels in middle-aged men have produced conflicting results, there is evidence that high sex hormone-binding globulin (SHBG) and androstenedione levels are more common among young adult African American men than white men [28]. Furthermore, it has been approved that the concentrations of androstenedione, testosterone, and progesterone were notably higher in African-American compared with Caucasian or Hispanic women [29]. The latter data are consistent with hypotheses that in-utero hormonal exposures may explain some of the ethnic group differences in cancer risk [30]. Moreover, it has been shown that the androgen levels in the Arab population are significantly lower than the levels in the Caucasian population [31].

1.3. Prostate pathology

The prostate is pathologically subdivided into 3 anatomical regions, with histologic compositions reflective of their respective functions. The anterior prostate makes up approximately 20% of the volume of the prostate and is composed of nonglandular fibromuscular stroma. The peripheral portion of the gland (commonly referred to as the peripheral zone) is comprised primarily of acinar tissue and forms the posterior and lateral surfaces of the prostate. In a normal prostate, the peripheral zone represents approximately 70% of the total glandular volume[32]. The vast majority of prostate carcinomas arise in the peripheral zone. The central

gland makes up the remainder of the prostate. The transitional zone is the periurethral tissue located in the central. The transitional zone hypertrophies with age and results in BPH [33](Figure 1-2 and Figure 1-3)[34].

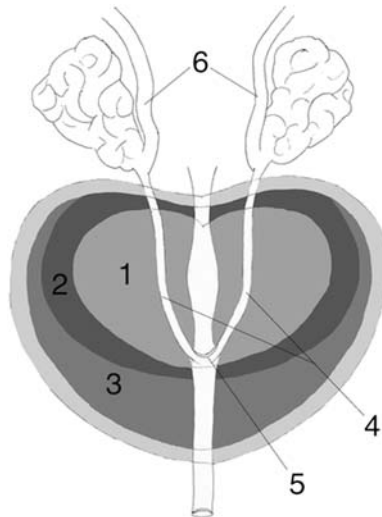


Figure 1-2: Prostate cross section (coronal): (1) transitional zone, (2) central zone, (3) peripheral zone, (4) ejaculatory ducts, (5) verumontanum, and (6) vas deferens.

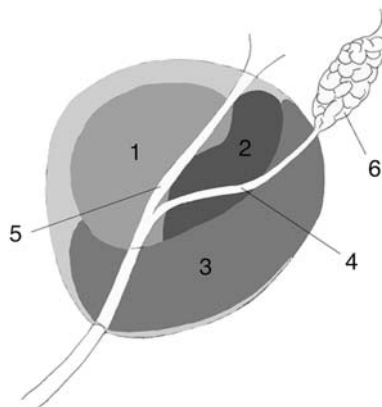


Figure 1-3.: Prostate cross section (sagittal): (1) transitional zone, (2) central zone, (3) peripheral zone, (4) ejaculatory ducts, (5) urethra, and (6) seminal vesicles.

Benign prostatic hyperplasia (BPH) is defined by proliferation of smooth muscle and epithelial cells in the transitional zone of the prostate, causing

gland enlargement and often resulting in lower urinary tract symptoms (LUTS). In men over 60 years of age, 50% have been diagnosed with BPH, and 75% of men over the age of 70 have 1 or more symptom attributable to BPH. By 85 years of age, 90% of men have symptoms of BPH [35]. BPH manifests clinically as LUTS, which include increased frequency of urination, nocturia, urgency, hesitancy, and weak urine stream. These symptoms are caused by disturbances in normal urine retention or voiding, which may be due to structural distortion and functional abnormality of the bladder neck, prostate, distal sphincter, or urethra. BPH alone is rarely a life-threatening condition but can significantly reduce the quality of life. The effect on quality of life is highly variable, and objective measures do not correlate well with the severity of symptoms [35, 36].

The most common primary prostatic malignancy is adenocarcinoma of the ductal or acinar epithelium [37]. The basal layer is normally absent and therefore there is infiltration into the prostatic stroma[38]. Macroscopically, the gland tends to be hard and white, though a soft mucin-producing variance exists. Less commonly, the transitional cell carcinoma (TCC) might primarily present in the prostatic urethra, or the prostate may be invaded by a primary TCC of the bladder [39]. Rhabdomyosarcoma of the prostate is rare but may be seen in childhood. Secondary deposits (metastases) from other sites are also rare [40].

About 75% of adenocarcinomas occur in the peripheral zone of the prostate and most (85%) are multifocal. 20% appear to arise from the transition zones and 5% from the embryologically distinct central zone [40, 41].

The tumour spreads locally through the poorly formed prostatic capsule into surrounding tissue, at which time it is termed as 'locally advanced'. Hence, the disease may involve the urethral sphincter, corpora of the penis, seminal vesicles, and trigone of the bladder including the distal ureters. Local spread is often along the course of autonomic nerves in the form of perineural invasion [42]. The most frequent sites of metastasis are lymph nodes and bone, although lung, liver, testis, and brain are not uncommon. Bone metastases are characteristically sclerotic, rarely lytic. The axial skeleton, which comprises the spine and pelvis, are most commonly affected, followed by the proximal long bones, ribs, clavicles, and the skull [40].

The Gleason grading system of prostatic carcinoma is the quintessential prognostic factor in predicting findings in radical prostatectomy, biochemical failure, local recurrences, lymph node or distant metastasis in patients receiving no treatment, radiation therapy, radical prostatectomy and other therapies, including cryotherapy and high intensity focal ultrasound therapy. Clinicians use various tools to predict outcomes, including the pathological stage or prognosis following radical prostatectomy or radiotherapy. All of these tools incorporate the Gleason score [43].

Donald F. Gleason in 1966 created a unique grading system for PCa based solely on the architectural pattern of the tumour, using a five-point scale, where patterns 1-3 represent tumours which most closely resemble normal prostatic glands and patterns 4 and 5 tumours show increasingly abnormal glandular architecture (Figure 1-4). An innovative aspect of this system, based on a study of 270 patients from the Minneapolis Veterans Administration Hospital, was that, rather than assigning the worst grade

as the grade of the carcinoma, the grade was defined as the sum of the two most common patterns and reported as the “Gleason score”[44].

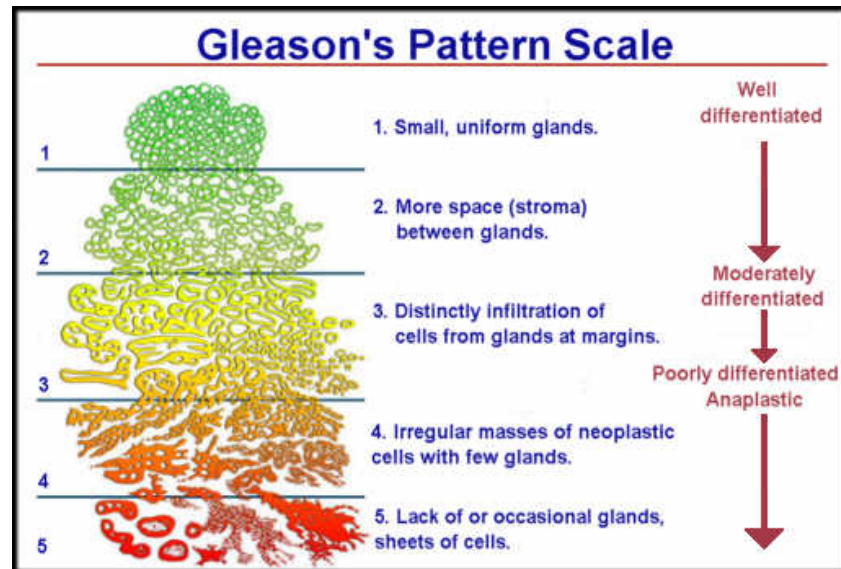


Figure 1-4: Gleason scoring grades .

(1) Gleason grade 1: Very well differentiated, small, closely-packed, uniform, glands in essentially circumscribed masses, (2) Gleason grade 2: Similar (to pattern 1) but with moderate variation in size and shape of glands and more atypia in the individual cells; cribriform pattern may be present, still essentially circumscribed, but more loosely arranged, (3) Gleason grade 3 : Similar to pattern 2 but marked irregularity in size and shape of glands, with tiny glands or individual cells invading stroma away from circumscribed masses, or solid cords and masses with easily identifiable glandular differentiation within most of them. (4) Gleason grade 4: Large clear cells growing in a diffuse pattern resembling hypernephroma; may show gland formation, (5) Gleason grade 5: Very poorly differentiated tumours; usually solid masses or diffuse growth with little or no differentiation into glands.

Staging involves determination of the anatomic extent or spread of a disease at the time of diagnosis based on clinical and pathologic criteria. Cancer's stage is based on the primary tumour's size and location and whether the tumour has spread to other areas of the body. The objective of

staging is to group malignancies with similar prognosis and therapeutical approach, to be able to compare clinicopathologic data from different institutions to perform clinical trials or studies on homogeneous patient populations. The TNM staging is the most widely used system for PCa staging and assesses the extent of primary tumour (T stage), the absence or presence of regional lymph node involvement (N stage), and the absence or presence of distant metastases (M stage) [45] (Table 1-1) [46].

Table 1-1: Definitions of clinical TNM

| Primary tumour (T) | |
|--------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------|
| TX | Primary tumour cannot be assessed |
| T0 | No evidence of primary tumour |
| T1 | Clinically inapparent tumour neither palpable nor visible by imaging |
| T1a | Tumour incidental histologic finding in $\leq 5\%$ of tissue resected |
| T1b | Tumour incidental histologic finding in $> 5\%$ of tissue resected |
| T1c | Tumor identified by needle biopsy (e.g. because of elevated PSA) |
| T2 | Tumor confined within prostate |
| T2a | Tumor involves one-half of one lobe or less |
| T2b | Tumor involves more than one-half of one lobe but not both lobes |
| T2c | Tumor involves both lobes |
| T3 | Tumor extends through the prostate capsule |
| T3a | Extracapsular extension (unilateral or bilateral) |
| T3b | Tumor invades seminal vesicle(s) |
| T4 | Tumor is fixed or invades adjacent structures other than seminal vesicles such as external sphincter, rectum, bladder, levator muscles, and/or pelvic wall |
| Regional lymph nodes (N) | |

| | |
|------------------------|--------------------------------------------|
| NX | Regional lymph nodes were not assessed |
| N0 | No regional lymph node metastasis |
| N1 | Metastasis in regional lymph node(s) |
| Distant metastasis (M) | |
| M0 | No distant metastasis |
| M1 | Distant metastasis |
| M1a | Non-regional lymph node(s) |
| M1b | Bone(s) |
| M1c | Other site(s) with or without bone disease |

The p53 tumor-suppressor gene is a transcription factor that regulates cell cycle progression, DNA repair, and apoptosis, thus serving as the checkpoint of cellular behaviour in response to DNA damage. The p53 gene is the most commonly mutated or deleted gene in human cancer [47, 48]. Although the reported frequency of p53 mutations in primary prostate cancer varies between 45% and 80%, most agree that p53 mutations are common in advanced prostate cancer [49, 50]. p53 protein expression in localized primary prostate carcinoma has been correlated with a higher Gleason grade and pathological stage. Also, in advanced prostate cancer, ample evidence points to an increased incidence of p53 mutations as a late event that correlates with progression, recurrence, and overall poor prognosis [51-54].

Loss of chromosome 9p21 harbouring the p16^{INK4a} tumour suppressor genes is frequently observed in human cancers. In prostate cancer, 9p21 loss is relatively rare (7-20%) in localized disease, but occurs more frequently in locally advanced disease and metastasized disease, and predicts unfavourable prognosis [55-57].

The role of p16^{INK4a} in prognosis prediction is different for patients treated with surgery when compared to irradiated patients. High levels of p16^{INK4a} in prostatectomy specimens predicted more rapid treatment failure, whereas decreased expression of p16^{INK4a} after radiotherapy predicted poor prognosis[58, 59]. The initial finding of p16^{INK4a} overexpression being associated with poor prognosis after prostatectomy may be explained by the fact that increased p16^{INK4a} expression is often tolerated by tumour cells carrying loss of other tumor suppressor genes such as Rb [60]. This seems likely, since p16^{INK4a} exerts a G1 block through inhibition of Rb and p16^{INK4a} expression is induced upon DNA damage[61]. Low p16^{INK4a} expression is often found in metastatic lesions[58, 60].

A normal functioning p16^{INK4a} mediated inhibition of Rb is required for a proper response to cellular stress such as oxidative stress and DNA damage[62]. Under normal circumstances, these conditions would lead to the induction of p53 and p16^{INK4a}, which would result in apoptosis or cell cycle arrest. Incorrect functioning of either of these axes may lead to the induction of cellular transformation and disruption of both pathways is required for cancer[63-67].

1.4. Human papilloma viruses (HPVs)

In 1933, Shope observed that the infection with the cottontail rabbit papillomavirus led to subsequent development of keratinous lesions, some of which progressed to invasive epithelial neoplasm's [68]. This observation led to the discovery of the first deoxyribonucleic acid (DNA) virus that caused tumours in mammals [69]. Papillomaviruses are highly specific for their respective hosts and have been shown to infect epithelial mucosa or cutaneous surfaces in almost all vertebrate species [70]. Infection results in primarily benign, self-limiting warts or epithelial tumours [71]. Extensive researches have linked some human

papillomavirus (HPVs) types to malignant and premalignant lesions of the uterine cervix, vulva, penis, conjunctiva, and upper aerodigestive tract [72-78]. HPV infections are associated with about 10% of the worldwide cancer burden, most of which are anogenital cancers, and HPV DNA is present in over 95% of cervical tumours worldwide [79-81].

HPVs belong to the Papovaviridae family, which includes the subfamilies papillomaviruses and polyomaviruses [82]. The family name of papovaviruses is a combination of papillomaviruses, polyomaviruses and vacuolating simian virus, the latter is an early name of SV40, a virus now included in the polyoma subfamily [71]. Papillomaviruses infect a broad spectrum of vertebrates, including humans [83]. They are highly species specific and do not infect over the species barrier [84]. Therefore, the different papillomaviruses are named after the species they infect, and they are numbered according to the order of their discovery [85]. The papillomaviruses that have been studied most extensively are cottontail rabbit papillomaviruses (CRPVs), bovine papillomaviruses (BPVs) from cattle, and human papillomaviruses (HPVs) [85-89]. So far, 85 different HPV genotypes have been fully characterized and approximately 30 additional putative genotypes have been partially described [88]. Initially, the definition of an HPV type was based on viral DNA re-association according to the liquid hybridization technique [87]. Later, in the early 1990's a novel HPV type was defined according to differences detected in the nucleotide sequences of the E6, E7 and L1 open reading frames (ORFs) of the HPV genome [85]. Since 1995 a new HPV type was defined when a sequence difference exceeded 10% in the L1 ORF, upon comparison with that of an established genotypes [89].

HPVs have a strict tropism for epithelial cells and are often divided into two sub-groups depending on which epithelial surface they most commonly infect [90]. There are mucosal HPV types and cutaneous HPV types, of which selected examples are listed in Table 1-1 [87].

Table 1-1 HPV type and associated lesions

| Subgroup | HPV infection type | Preferentially found in |
|-----------|-------------------------------------------------|----------------------------|
| 1 | Plantar warts | Cutaneous |
| 2,4 | Common warts | Cutaneous |
| 5 | Benign/ malignant verrucae lesions | Cutaneous |
| 6,11 | Condyloma, laryngeal papilloma | Mucosal, low-risk |
| 7 | "Butcher's warts", oral papilloma (HIV patient) | Mucosal , cutaneous |
| 16 | Anogenital cancer and precursors | Mucosal, high-risk |
| 18 | Anogenital cancer and precursors | Mucosal, high-risk |
| 19-25 | Epidermoid dysplasia verruciformis lesions | Cutaneous |
| 30 | Laryngeal carcinoma | Mucosal |
| 31,33 | Anogenital cancer and precursors | Mucosal, intermediate-risk |
| 42,43,44 | Anogenital benign lesions | Mucosal, low-risk |
| 45,56 | Anogenital cancer and precursors | Mucosal, high-risk |
| 51-52, 58 | Anogenital cancer and precursors | Mucosal, intermediate-risk |
| 53-55 | Anogenital benign lesions | Mucosal |
| 70 | Vulvar papilloma | Mucosal |
| 72,73 | Oral papilloma (HIV patient) | Mucosal |
| 80 | Normal skin | Cutaneous |

HPVs can be divided into so called low-risk, intermediate risk and high-risk HPV types, depending on if they are commonly isolated from benign or malignant cervical lesions [91]. HPV types that are found commonly in anogenital and other cancers are 16, 18, 31, 33, and 5 [87]. HPV types 39, 5, 51, 52, 56, 57, 58, 59, and 61 may also be present less frequently in dysplastic and malignant pathologies of other anogenital sites [89]. HPV 16 is the most commonly found in squamous cell cancers of the cervix, whereas HPV18 predominates in adenocarcinomas of the cervix [78, 91]. The molecular basis for the tissue specific distribution of these two high-risk HPV types is still not clear [92]. The low-risk HPV types include HPV6, 11, 13, 32, 34, 40, 42, 44, 53, 54, 55, and 63, which are usually

associated with benign lesions such as cervical condylomas [89, 93, 94]. High-risk HPV types can induce increased chromosomal abnormalities and aneuploidy in the infected cell and encode oncoproteins (E6, E7) that interact specifically with the cellular proteins (*p53*, *Rb*) which are responsible for the control of cell growth and proliferation [95]. Because high- and low-risk HPV categorising primarily emerges from studies of cervical neoplasia, it is likely that additional HPV types, which are found less frequently in other malignant tissues, may also share some of the properties with the high-risk HPV types [96]. This is almost true with the anal cancers [97]. Sequence comparisons between high-risk and low-risk HPV types have consistently revealed a single amino acid sequence difference at residue 21, for example; Asp 21 in HPV16 E7 protein corresponds to a glycine residue in low-risk HPV6 E7 [98]. Consequently, given the level of intragenomic variability within an HPV type, single amino acid substitutions in low-risk HPV E7 proteins may promote features normally characteristic of high-risk HPV E7 oncoprotein [99].

1.5. Viral particle and its genomic organisation

HPVs are non-enveloped, small circular double-stranded DNA viruses with an icosahedral capsid [100]. The capsid is made of 72 capsomers and it is approximately 55 nm in diameter [101].

The DNA of HPV is chromatin-like and covered by histones [86]. Virus particles contain a circular, double stranded, covalently closed DNA of about 8 kilo base pairs in length [101, 102]. The viral genome is organised into three general segments of unequal size (Figure 1-5) [89]. About 10% of the genome is contained within the long control region (LCR) and functions in the regulation of viral gene expression. The remainder of the genome is organized into early (E) or late (L) genes, encoded by 50% and

40% of the genome, respectively [103, 104]. All viral genes are encoded on one strand of DNA [105].

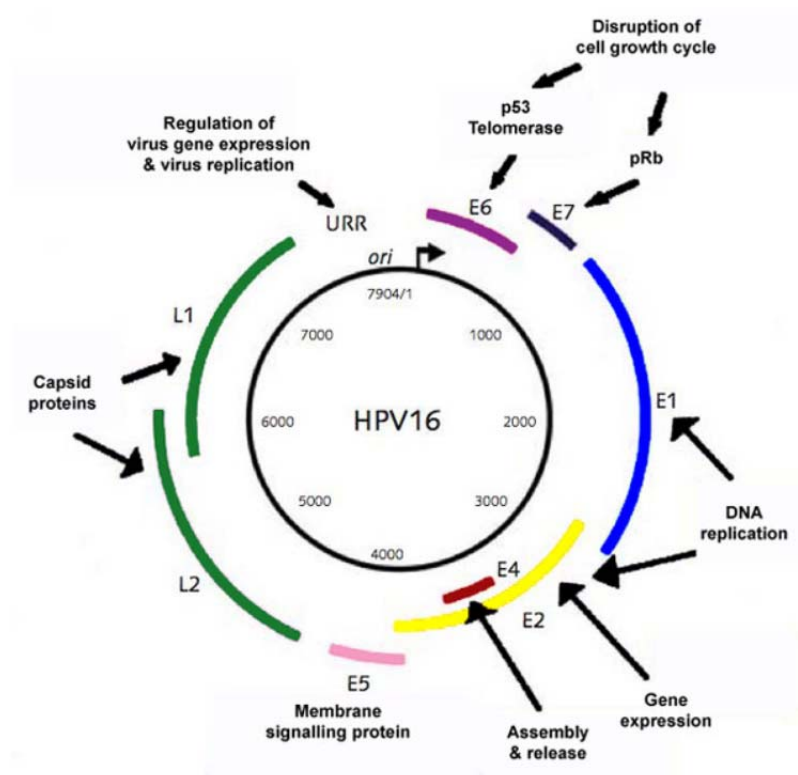


Figure 1-5: Genomic organisation of HPV 16 .

1.6. Viral genes and proteins

The genome is divided into three regions; the long control region (LCR) and the two open reading frames ORFs: the early (E) and the late (L) region [105]. The LCR is a non-coding region where the regulation of viral gene expression is controlled [95]. The early open reading frames (ORFs); (E1, E2, E4, E5, E6 and E7) encodes for proteins involved in regulatory functions and DNA replication [96], while the late ORFs (L1 and L2) encodes for proteins that constitute the viral capsid [104] (Table 1-2). The early genes E3 and E8 are found in some papillomaviruses, but not in HPVs [106].

Table 1-2 HPV proteins [96].

| Protein | Size | Interactions & functions | Cellular location | Expressed in |
|------------------|-----------|-------------------------------------------------------------------------------------------------------------------------------------------------------------|------------------------------------------|--------------------------------------------|
| Early (E) | | | | |
| E1 | 68-85 kDa | -DNA binding -ATPase and helicase activities -Initiation of viral replication | Nucleus | Basal and differentiating epithelial cells |
| E2 | 43-48 kDa | -DNA binding -Regulation of viral transcription and replication | Nucleus | Basal and differentiating epithelial cells |
| E4 | 17-16 kDa | -Interacts with keratin networks -Maturation and release of viral particles -Regulating gene expression, Gs arrest | Cytoplasm | Differentiating epithelial cells |
| E5 | 44-91 aa | -Interacts with EGF receptor | Golgi apparatus, ER* and plasma membrane | Differentiated epithelial cells |
| E6 | 16-18 kDa | -Interacts with E6-AP -Gene transcription -Prolonging of cellular lifespan -Up-regulation of telomerase activity | Nuclear matrix | Basal and differentiating epithelial cells |
| E7 | 14-21kDa | - Interacts with pRb proteins, cyclin A and E, p21, p27 and HDACs ² - Induces cellular proliferation, - Immortalization and transformation | Nucleus | Basal and Differentiating epithelial cells |
| Late (L) | | | | |
| L1 | 55 kDa | - Self-assembles into viral particles - Large capsid protein, 360 copies/viral particle | Nucleus | Terminally Differentiated epithelial cells |
| L2 | 76-78kDa | - Interacts with L1 and E2 - Small capsid protein, 12 copies/viral particle - Relocalizes L1 and E2 to PODs** | Nucleus | Terminally Differentiated epithelial cells |

*ER = endoplasmic reticulum, ²HDACs, histone deacetylases, ** = promyelocytic oncogenic domains (PODs).

1.7. E1 protein of HPVs

The E1 ORF is the largest and most highly conserved of all HPV ORFs and codes for a polycistronic RNA, which is translated into 68-85 kDa proteins [107]. The E1 protein is expressed at low levels in HPV positive cells and has site-specific DNA binding sequences that bind, weakly, to

the origin of replication and initiate DNA replication [86, 96]. DNA binding is stabilized by complex formation with the E2 protein [96] and once bound, E1 forms hexamers with high binding affinity for DNA [108]. Without E1, there would be no viral DNA replication, since E1 is essential for its initiation [107]. Also, E1 is regulated through interactions with cyclin A and cyclin E by cyclin-dependent kinase-mediated phosphorylation [107], resulting in inactivation of the E1 nuclear export sequence (NES), keeping E1 in the nucleus [109], where it can initiate viral DNA replication [102].

1.8. E2 Protein of HPVs

The E2 ORF encodes for three proteins, which all act as transcription factors [86, 96]. These proteins have a DNA binding region in their C-terminal and regulate viral transcription and replication by forming dimers at specific binding sites [100, 110]. The E2 proteins are essential for viral replication, since it directs E1 to its DNA binding sites and enhances the binding affinity of E1 to DNA [96, 107].

It has been suggested that E1 and E2 control the copy number of episomal HPV copies since the number of HPV copies increases when E1 or E2 expression is increased [111]. The E2 ORF is frequently disrupted when the HPV genome is integrated. This leads to a more malignant phenotype as seen in cervical biopsies and cell lines derived from cervical cancer [112]. This phenomenon has been attributed to the loss of the E2 repression of the E6 and E7 transforming proteins [100, 113].

1.9. E4 Protein of HPVs

The E4 ORF is expressed in low amounts early in the life cycle of the virus, however, upon epithelial cell differentiation, the expression of E4 increases, and E4 is then the most highly expressed HPV protein [114]. The E4 ORF is translated from spliced transcripts together with the five first amino acids of E1, resulting in an E1^{E4} fusion protein, where the E1 sequence is used for initiation of translation [115]. E4 proteins are exclusively found in the differentiating layer of the infected epithelium [86, 96]. High risk HPV E4 was suggested to be involved in facilitating release of viral particles [96], because E4 interacts with the keratin networks and causes their collapse. Furthermore, E4 may play a role in regulating gene expression, as it showed the ability to induce G2 arrest in vitro in a variety of cell lines [96, 115].

1.10. E5 Protein of HPVs

The E5 ORF encodes for a small highly hydrophobic membrane bound protein, which is lately expressed in the virus life cycle in differentiated epithelial cells [96, 116]. The E5 protein of high risk HPVs has weak transforming activities [96, 117], while the corresponding E5 protein in bovine papillomavirus (BPV) is the major transforming protein [118]. BPV E5 and HPV E5 do not share sequence homology and while BPV E5 acts through interactions with the platelet-derived growth factor (PDGF) receptor, the HPV E5 is proposed to interact with the epidermal growth factor (EGF) receptor [116, 118]. When HPV E5 is over expressed in the infected cells, it is generally accompanied by a considerable increased phosphorylation process of the EGF receptors [119].

1.11. E6 protein of HPVs

The HPV E6 protein is a relatively small polypeptides of approximately 150 amino acids and contain two zinc-finger motifs each of which consists of 2 CXXC sequences separated by 29 amino acids that each are related to the carboxyl terminal domain of E7 [120], whose integrity is essential for E6 function [121]. The E6 ORF encodes for a basic protein, which has two zinc-binding motifs in the C-terminal [122]. The HPV E6 lacks any intrinsic enzymatic and specific DNA-binding activities and functions by subverting host cellular protein complexes [123]. The E6 protein of high risk HPVs is classified as an oncoprotein and can transform human mammary cells [124, 125] and cooperate with E7 in transforming human cells [126]. E6 is expressed early after viral infection and facilitates viral production by inducing several cellular changes, and by prolonging of cellular lifespan through blocking of the cellular apoptosis pathway [96, 125]. HPV E6 interacts with a ubiquitin ligase and forms complexes with *p53*, which is then degraded through ubiquitin dependent mechanisms [127]. Moreover, E6 can block translocation of *p53* into the nucleus [128] and thereby inhibits the gene regulatory functions of *p53* [128, 129]. The consequence of *p53* degradation and blocking of *p53* move into the nucleus is the disruption of *p53* mediated cell cycle control, which means that the cell can continue to divide despite the DNA damage [96, 128].

The most studied biological activity of HPV16 E6 is the ability to associate with *p53* and the ubiquitin ligase E6-AP, which leads to the ubiquitin-mediated proteasomal degradation of the *p53* tumour suppressor [130]. The *p53* tumour suppressor senses cellular stress including aberrant S-phase entry induced by expression of the HPV E7 oncoprotein. The activation of *p53* results in transcriptional induction of abortive cellular

programmes such as G1 growth arrest and apoptosis, which have been collectively referred to as the trophic sentinel response [131]. Inactivation of *p53* by the E6 protein presumably serves to abrogate this response, thereby allowing for persistent S-phase competence in differentiated cells [132]. HPV E6 expression also increases transcription of the catalytic subunit of telomerase, hTERT, which contributes to cellular immortalisation of primary human epithelial cell lines [133].

High-risk HPV E6 proteins contain a short peptide sequence (S/T)-X-V-I-L at their carboxyl termini, which mediates association with cellular PDZ proteins [134]. The integrity of the PDZ binding sequence on E6 is important for the HPV viral life cycle, as well as the transforming activities of E6 [135]. Many PDZ proteins act as molecular scaffolds and regulate a number of important processes including cell polarity [136]. It is not clear whether high-risk HPV E6 proteins associate with one single PDZ protein or whether they can target multiple family members, since a number of different candidates have been reported [137]. Since PDZ protein and E6-AP binding on E6 are not mutually exclusive, E6-associated PDZ proteins can be targeted for ubiquitination by E6-AP [138].

Many papillomaviruses E6 proteins associate with paxillin, and association with E6 leads to disruption of paxillin's association with the focal adhesion proteins vinculin and focal adhesion kinase (FAK). This causes defects in the cellular actin cytoskeleton, as is typically observed in transformed cells [139].

1.12. E7 Protein of HPVs

The E7 ORF encodes for a 98 amino acid phosphoprotein that is actively transported to the nucleus, which has two zinc-binding motifs in the C-

terminal half of the protein. These motifs are essential for proper protein folding and stability [100, 140]. The E7 protein has been detected in both the nucleus and the cytoplasm and shown to induce cellular proliferation, immortalization and transformation [111, 127, 141].

HPV16 E7 lacks any known intrinsic enzymatic activities and does not specifically associate with DNA sequences. Rather, it functions by associating with and functionally modifying host cellular regulatory protein complexes [142]. The high -risk HPV E7 can immortalize human cells, by interactions with factors involved in the control of the cell cycle [140, 143]. One of these interactions is with the proteins of the *Rb* family [113, 125]. Binding of E7 to p*Rb* results in release of the E2F transcription factor, which leads in turn to the activation of gene transcription factor p16^{INK4a} [114]. In addition, high risk HPV E7 mediates degradation of p*Rb* [144]. The *Rb* proteins play an essential role in cell cycle regulation promoting the transition from G1 into S-phase. In normal cells, *Rb* is hypophosphorylated in early G1 and becomes increasingly phosphorylated towards S phase. In its hypophosphorylated form, *Rb* binds E2F transcription factors and actively represses transcription from promoters containing E2F sites [96]. By binding *Rb* in a hypophosphorylated state, E7 prevents it from binding to E2F and thereby promoting cell cycle progression [96, 145].

The ability of E7 to bind to p*Rb* is shared between the high and low risk HPVs, although the interaction between high risk HPV E7 and p*Rb* is much stronger [143]. Furthermore, E7 proteins also interact with the histone deacetylases (HDACs) which are normally recruited to the E2F inducible promoters by p*Rb* and represses the E2F function [96, 125].

Given its subcellular localization, HPV16 E7 has been shown to associate with nuclear as well as cytoplasmic cellular target proteins [142]. HPV16 E7 shares sequence similarity to a small portion of conserved region 1 (CR1), as well as the entire conserved region 2 (CR2) of the adenovirus (Ad) E1A oncoprotein [146]. The CR2 homology domain contains the pRb tumour suppressor core-binding site, LXCXE (L, leucine; C, lysine; E, glutamic acid; X, any amino acid residue), as well as a casein kinase II phosphorylation site [140]. The carboxyl terminus consists of two CXXG motifs separated by a 29 amino acid spacer region that forms a novel zinc binding structure [140]. Unlike Ad E1A and SV40, which target pRb through a stoichiometric mechanism, HPV16 E7 inactivates this tumour suppressor protein through ubiquitin-mediated proteasomal degradation, and sequences within the HPV16 E7 CR1 homology domain that do not contribute to pRb binding are necessary for this activity [134, 140, 147]. These HPV16 E7 sequences serve as a binding site for a cullin 2 containing ubiquitin ligase complex, which contribute to pRb degradation [146]. In addition, the CR1 homology domain also contains a binding site for the N-end rule ubiquitin ligase p600 [148], which appears to play an important role in regulating anoikis, a form of apoptosis that is induced upon detachment of cells from a substratum [149]. The carboxyl terminus of HPV16 E7 contains additional sequences that are necessary for transformation [147]. While a large number of putative cellular targets, including histone-modifying enzymes that bind to carboxyl terminal sequences have been identified, it is not clear which of these interactions, if any, contribute to cellular transformation [150].

HPV16 E7 has been shown also to associate with pyruvate kinase, and modulate the activity of alpha glycosidase and induce a metabolic switch from oxidative phosphorylation to glycolysis [151]. In addition, HPV 16 E7

expression causes aberrant activation of the survival kinase AKT in differentiating keratinocytes, which has been linked to increased cell motility [152]. In addition, HPV16 E7 has been shown to cooperate with E6 to induce expression of proteins that modules angiogenesis [147].

1.13. L1 Protein of the HPVs

The L1 ORF is highly conserved between different HPV types [103, 104]. It encodes for the major capsid protein, which is present in 360 copies per virion [96]. The L1 protein self assembles into pentamers [153], which are the building blocks of the viral capsid and they can also self-assemble into virus-like particles (VLP) [102].

1.14. L2 Protein of the HPVs

The L2 ORF encodes for the minor capsid protein, which is present in around 12 copies per virion [154]. The L2 protein is highly phosphorylated and it is required for encapsidation of viral DNA into the capsid, and it acts through relocalization of the L1 protein to sub-nuclear domains called promyelocytic oncogenic domains (PODs) [155]. In addition, the L2 protein also guides the E2 protein to the PODs and thus helps viral assembly and viral genome packaging [155]. Together L2 and L1 proteins can self-assemble into VLPs [156].

1.15. Human papilloma viruses (HPVs) life cycle

HPVs comprise a large and diverse group of viruses with over than 100 subtype [85]. HPVs infect epithelial cells and all require terminal differentiation of the host infected cells to produce infectious virions [89]. For an active infection to occur, HPVs must gain access to the stem cells of basal layer of the epithelium through an abrasion or micro-wounds

[151]. For example, HPV types 1 and 2 need access to the stem cells of the cutaneous basal epithelium, while types 6 and 16 should have access to the stem cells of the mucosal basal epithelium [89, 102, 157]. The virus then enters the host cell through a mechanism that is not fully understood, but probably involves initial binding to heparin sulphate on the cell surface followed by receptor binding and viral uptake, although there is controversy regarding the identity of the virus receptor [102]. Following viral uncoating and transport to the nucleus, an initial surge of replication brings the viral genome to a copy number of between 50 to 100 per cell as this copy number is type dependent [89, 151]. The viral genomes are maintained episomally at these low copy numbers in the nuclei of infected cells for a long period, which may last for decades in the basal epithelium [151, 158]. During that period, the virus replicates in synchrony with the S-phase of the host cell [89, 90, 151].

However, the productive phase of the viral life cycle, which includes HPV genome amplification, production of capsid proteins, and packaging of newly synthesized genomes, occurs exclusively in the terminally differentiated cells where they can remain infectious over extended period [159].

HPVs encode two proteins, E1 and E2, which directly contribute to the viral genome replication [160]. The E1 origin-binding protein is the only virally encoded enzyme and has intrinsic ATPase and helicase activities [108]. E1 forms a complex with the E2 protein, the major HPV-encoded transcriptional regulatory protein [161]. E2 binds with high affinity to specific DNA sequence AGCN₆GGT in the viral regulatory region, whereas E1 binds to the AT-rich replication origin sequences with relatively low affinity [162]. The origin sequence is often flanked by E2 binding sites resulting in high affinity binding of the E1/E2 complex to the origin of replication [163].

Viral gene expression is controlled by the long control region (LCR), which contains viral promoter and enhancer sequences and the origin of replication. The LCR is also commonly called the upstream regulatory region (URR) or the non-coding region (NCR) [86, 100]. The complex regulation of viral gene expression is controlled by both cellular and viral transcription factors [86, 100]. Examples of cellular transcription factors that bind to the LCR are NF-1, AP-1, Oct-1, TEF-1, TEF-2, SP-1 and YY-1 [85, 86, 164]. Dysregulation of these transcription factors seems to be of importance for the carcinogenesis process in HPV-induced lesions [86].

The regulation of viral gene expression is a complex multifactorial process, which involves both viral and host factors [85]. In the basal cells of the squamous epithelium low levels of early transcripts (E6, E7, E5, E1 and E2) are detected [107]. These transcripts are initiated at the early promoter, located upstream of the E6 ORF, in the LCR [165].

With the exception of the E1/E2 origin-binding complex, HPVs do not encode enzymes that are necessary for viral genome replication and co-opt the host DNA synthesis machinery [89]. It requires the cellular DNA machinery including DNA polymerase α /primase, DNA polymerase δ , replication protein A, PCNA and topoisomerases [109]. Because high-copy number HPV genome replication and viral progeny synthesis is limited to terminally differentiated cells, which are growth arrested and incompetent for DNA replication, a major challenge for the viral life cycle is to re-establish and sustain a replication environment in these cells [90, 96, 150]. To achieve that task, the HPV E7 protein acts to induce and maintain of S-phase competence in differentiating epithelial cells through a variety of mechanisms [150]. The most important of the latter is when

the HPV E7 proteins bind to the retinoblastoma tumour suppressor protein *pRb* and the related *p107* and *p130* pocket proteins [166]. These proteins have been involved in regulating the G1/S phase transition through the E2F transcription factors [167]. The G1 specific *pRb*/E2F complex is a transcriptional repressor that inhibits the S-phase entry [168]. In normal cells, *pRb* is phosphorylated by cycling/CDK complexes in late G1, the *pRb*/E2F complex dissociates, and DNA-bound E2Fs act as transcriptional activators [169]. The *pRb*/E2F re-forms when *pRb* is dephosphorylated at the end of mitosis [168]. This regulatory loop is subverted by HPV E7 proteins, which can associate with *pRb* and abrogates the inhibitory activity of *pRb*/E2F complexes [170]. E7 proteins encoded by the low-risk HPV associate with *pRb* in a lower affinity than high-risk HPV E7 proteins [171]. Additionally, high-risk HPV E7 proteins induce proteasome-mediated degradation of *pRb* [126]. Moreover, E7 protein abrogate the action of Cyclin-dependent kinase inhibitors (CKIs) *p21*(Cip1) and *p27*(Kip1), which regulate cell cycle withdrawal during epithelial cell differentiation, thereby uncoupling epithelial cell differentiation and cell cycle withdrawal [172]. This leads to the formation of hyperplastic lesions, warts and is necessary for the production of progeny virus [173].

In addition, HPV E6 interacts with a ubiquitin ligase called E6-AP, which forms complexes with *p53*. This leads to rapid degradation of the *p53* protein. In these conditions *p53* mediated cell cycle control is disturbed and the cell continues to divide in spite of DNA damage [174]. HPV E6 also confers prolonged life span by up-regulation of the telomerase activity [175] (Figure 1-6) [111].

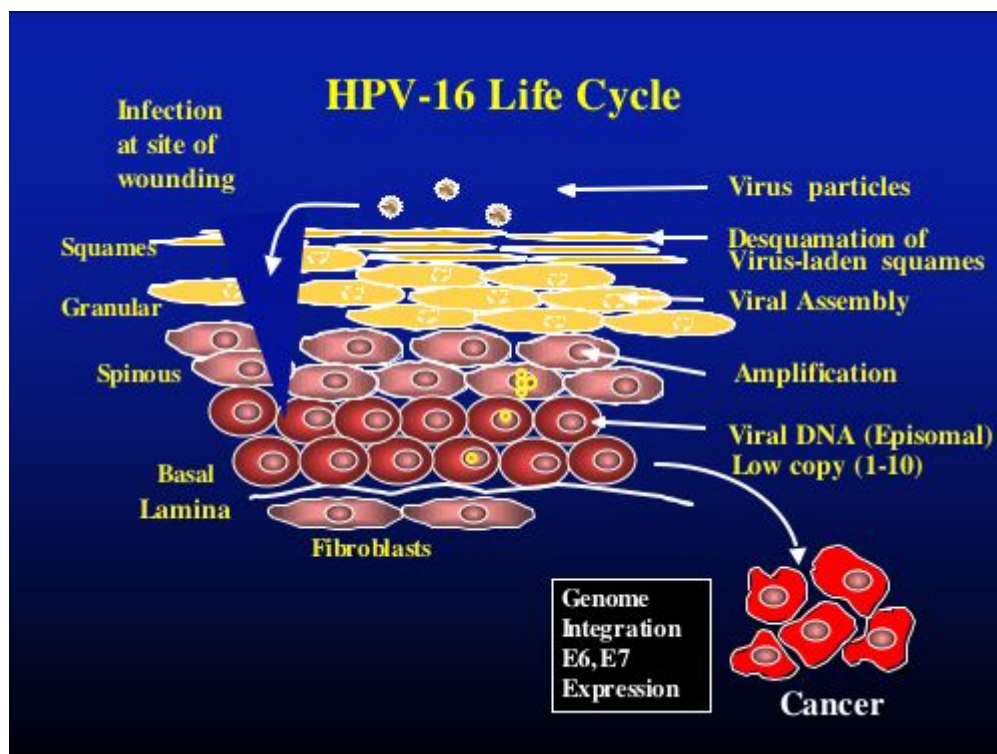


Figure 1-6: HPV16 life cycle in cervical cancer.

1.16. Viral Characteristics

The HPV viruses are present in a latent form in proliferating cells, while the productive large-scale viral DNA replication, translation and the functional activities of the late proteins as well as the viral assembly are restricted to the differentiating layers of skin and mucosa [86].

In mucosal HPV-associated tumours, high-risk viral DNA occurs in the tumour cell at a minimum of one viral copy per cell genome, is transcriptionally active, and is often, although not always, integrated [176]. However, in cervical cancers, a proportion of the cancers have only episomal HPV-DNA [177], and in skin cancer associated with epidermodysplasia verruciformis, the HPV genome remains episomal [178]. In very rare instances, such as squamous cell carcinomas in individuals with recurrent respiratory papillomatosis, low-risk HPV

types 6 and 11 may be found in tumours [176]. Thus, in cancers hypothesized to be associated with mucosal HPV, it might be expected that the genome of high-risk HPV (types 16, 18, 31, 33, 35, 39, 45, 51, 52, 55, 56, 58, 59, 68, 73, 82, and 83) to be localized in tumour cell nuclei and to express viral oncogenes [88]. The presence of the viral genome in a variety of diseases, starting from premalignant lesions to carcinoma *in situ*, invasive disease, and metastatic cells, might hint to the importance of the virus in the initial promotion and maintenance of different malignant phenotypes [176].

The one-to-one tumour cell-virus relationship may not hold for all papillomavirus-associated tumours. For instance, in bovine alimentary tract cancers associated with bracken exposure and bovine papillomavirus type 4 (BPV-4) infections, the viral genome is lost from the tumour cells but is present in the benign papillomas from which they arise [176]. Although the hit-and-run role in genital HPV types in human tumours still controversial, it has been hypothesized for skin cancers [176, 179]. Investigating this possible mechanism in humans would be difficult, since it would require an exhaustive search for the virus in premalignant lesions that are often subclinical [176, 179]. Such a hit-and-run role for the virus can be suggestible when there is a strong association, after adjustment for possible confounding factors, between HPV L1 seroreactivity and cancer risk in the absence of detectable HPV-DNA in tumours [176, 179]. There are as yet no consistent reports of such an association in the literature [180].

Detection of the genomic DNA by highly sensitive polymerase chain reaction (PCR) techniques alone is insufficient evidence for the presence of pathogenically significant virus [176]. However, a detailed study of a

single tumour can provide compelling preliminary evidence for the HPV aetiology of that tumour type such as testing the pathological effects of the virus on the apoptosis pathway in host genome [181].

1.17. The role of HPV oncoproteins in inducing genomic instability

HPV- related cancers generally develop years or decades after the initial infection, and these tumours have suffered a multitude of genomic aberrations [182]. The acquisition of some of these genomic aberrations appears to define certain stages of disease progression [183]. The action of the high-risk HPV E6 and E7 oncoproteins on telomerase activity and the *p53* and *pRb* tumour suppressors are sufficient to lead to extended uncontrolled proliferation and cellular immortalisation, but acquisition of additional host genome mutations is necessary for malignant progression [184]. A defining biological activity of high-risk HPV E6/E7 proteins is their ability to subvert genomic integrity [185]. Hence, high risk HPV E6/E7 oncoproteins not only contribute to initiation but also play a key role in malignant progression [186].

There are two principal mechanisms that lead to genomic instability; Subversion of cell cycle checkpoint mechanisms and DNA repair pathways can lead to perpetuation of mutations induced by environmental triggers such as UV irradiation or exposure to chemical compounds that cause DNA damage [187]. Alternatively, genomic instability can be triggered by active mechanisms that cause genomic destabilisation, which have been collectively referred to as mutator phenotype [188]. Expression of HPV E6/E7 oncoproteins causes genomic instability by both of these mechanisms [134].

HPV16 E7 has activities of mitotic mutator and its expression in primary human epithelial cells causes several types of mitotic abnormalities [189].

These include induction of supernumerary centrosomes, lagging chromosomes, and anaphase bridges [189]. Of these, induction of supernumerary centrosomes has been studied in detail [190]. Centrosome-associated multipolar mitosis is histopathological hallmark of high-risk HPV-associated cervical lesions. HPV16 E7 induces centrosome abnormalities through multiple, cooperating pathways. HPV16 E7 expression causes aberrant activation of CDK2 through several mechanisms, including E2F-mediated transcriptional activation of expression of the CDK2 catalytic subunits cyclins E and A as a consequence of pR/p107/p130 degradation and inactivation of the CDK2 inhibitors p21^{CIP1} and p27^{KIP1} [191]. Induction of supernumerary centrosomes by HPV16 E7 is strictly dependent on CDK2 activity [192]. In addition, HPV16 E7 associates with the centrosomal regulatory protein gamma-tubulin and inhibits its loading on centrosomes [192, 193]. Consequently, the process of centrosome duplication is uncoupled from the cell division cycle; result in the synthesis of multiple daughter centrioles from a single maternal centriole template [140]. Whereas expression of HPV16 in primary cells effectively induce centrosome overduplication, E6 co-expression is necessary for induction of multipolar mitoses [194].

HPV16 E7 expression also causes a higher incidence of DNA double strand breaks, which can lead to breakage of fusion bridge cycles and chromosomal translocations [150, 194]. Specific recurrent chromosomal translocations are well documented drivers of haematological malignancies and, more recently, similar translocations have also been documented to contribute to the genesis of human solid tumours [195]. Chromosomal translocations are regularly detected in cervical cancer

specimens. The mechanistic basis of the formation of mitosis with lagging chromosomal material still awaits full investigation [196].

The ability of the HPV16 E6 protein to contribute to genomic destabilisation is to large part based on its ability to inactivate the *p53* tumour suppressor. Therefore, the DNA damage-induced G1/S checkpoint is non-functional, and HPV16 E6-expressing cells also exhibit mitotic checkpoints defects [150]. HPV16 E6-mediated *p53* degradation also causes subversion of a post mitotic checkpoint that is specifically triggered in cells when they re-enter a G1-like state after a failed mitosis [197]. Such cells have a tetraploid rather than the normal diploid set of chromosomes and contain two centrosomes rather than one. Cells with *p53* defects will disregard this checkpoint, re-enter S-phase, and may eventually undergo tetrapolar mitosis, which can lead to generation of aneuploidy progeny. Consistent with this model, HPV16 E6 expression cells show marked nuclear abnormalities [130, 150, 189].

In addition to passive mechanisms of cell cycle checkpoint subversion, HPV16 E6 may also contribute to genomic destabilisation through active mutator mechanisms. HPV16 E6 has been shown to associate with single-stand DNA break repair protein XRCC1 and induce degradation of O6-methylguanine-DNA methyltransferase, which also involved in single-stand DNA break repair [198]. It has also been reported that HPV16 E6 expression decreases the fidelity of DNA end joining [199]. Moreover, while HPV16 E6 expression does not induce centrosome over-duplication, it greatly increases the incidence of multipolar mitoses in cells that contain supernumerary centrosome due to HPV16 E7 expression [186, 187].

1.18. Molecular mechanisms of high-risk HPV-mediated cellular transformation in cervical cancer

The transforming activities of high-risk HPVS reflect the necessity of the virus to establish and maintain a replication-competent cellular milieu in terminally differentiated cells [200]. Three high-risk HPV proteins E5, E6, and E7- have been shown to exhibit transforming activities in cellular and animal systems [150, 200]. However, only E6 and E7 are regularly expressed in cervical carcinoma where the HPV genome is frequently integrated into a host chromosome [134]. The integration event is relatively nonspecific with the host chromosome [143]. Integration frequently involves common fragile sites [201], and in some cases, HPVs integrate in the vicinity of cellular proto-oncogenes like *c-myc* [202]. However, integration of high-risk HPV genomes often causes disruption and sometimes deletion of the E2 ORF, which encodes a transcriptional repressor of E6/E7 expression [203]. Moreover, HPV16 E6/E7 mRNAs are more stable when expressed from integrated copies as compared to episomal HPV16 genomes. Thus, E6, E7 oncoprotein expression is deregulated in cervical carcinomas [123]. Ectopic expression of HPV E6 and E7 in primary human epithelial cells facilitates immortalisation, and when high-risk E6/E7 expressing cells are grown under conditions where they can form multi-layered, skin-like structures, they can exhibit cellular abnormalities that are reminiscent of high-grade premalignant cervical lesions [204]. Nonetheless, low-passage high-risk HPV immortalised cells are non-tumourigenic in immune-deficient mice but can undergo full transformation upon prolonged passaging [205], or when additional oncogenes such as *ras* or *fos* are expressed [150]. As such, this mimics the situation in vivo, where high-risk HPV-associated cervical lesions progress to cervical cancer after long-term persistent infection [123, 150]. Cervical cancers show hallmarks of chromosomal instability and are generally

aneuploidy [182]. Cervical cancers that arise upon expression of HPV16 E6/E7 in basal epithelia from keratin K14 promoter in transgenic animals require chronic treatment with low doses of oestrogen, further supporting the notion that additional genomic aberrations are necessary for cancer development [206]. Nonetheless, high-risk HPV E6/E7 oncoprotein directly contribute to malignant progression through induction of genomic instability [185]. Moreover, persistent HPV E6/E7 expression is necessary for maintenance of the transformed phenotype of HPV positive cervical cancer lines [186].

The polymorphism at codon 72, which encodes for arginine or proline, of the *p53* tumour-suppressor gene, in cervical cancer cases has been discussed as a possible determinant for cervical cancer risk [207]. Also, it has been suggested that the arginine form at codon 72 of *p53* was more susceptible to E6-mediated degradation than the proline form in actively infected cells with high risk HPVs [208]. Furthermore, it has been found that cervical cancer patients who are homozygous for the arginine form at codon 72 were approximately sevenfold more susceptible to HPV-associated tumorigenesis than heterozygotes [209].

It has been found that the apoptosis index using *in situ* apoptosis TUNEL assay was low in normal epithelium of cervical cancer cases but increases with the grade of the tumour [210]. This contradicts completely with the results reported from the morphological criteria of apoptosis with H&E staining using light microscopy [211]. Therefore, it has been concluded that the *in situ* apoptosis TUNEL assay indices might be useful marker to follow up the development of cervical cancer cases [211].

1.19. HPVs infections and prostate cancer

Almost 29 papers between 1990 until March 2010 have studied HPVs in prostate tissues using mainly southern blotting and/or PCR using six primers at most [212]. In nine papers HPVs have not been found either in BPH or PCa cases, whereas in the other 20 papers the detection rate ranged from 2% to 63% [212]. Most of the researches, which did not find HPVs infections in PCa and BPH cases, were conducted in Western Europe [212].

The hypothesis of HPVs infection involvement in prostate cancer development was controversial over many years when different researchers came up with different conclusions. For example; Leiros GJ *et al.* (2005) could establish the presence of HPV infection in 41.5% cases of prostate cancer cases in 41 studied cases by using PCR technology [213]. However, May M *et al.* (2008) could not establish any correlation between the HPV infection and the biopsies from prostate cancer patients using PCR in 213 cases [214]. These differences may be attributed to the variations in the level of infections with HPVs among nations, in addition to the incompatibility the different sensitivities of the rigorous laboratory tests used by the researchers [212].

Transmission of anogenital HPV16 infections have been consistently associated with sexual contact and the prevalence of cervical HPV infections increases between the ages of 15 and 40 years especially among women with multiple sexual partners [86, 103, 215]. Modes of transmission of HPV infection to the prostate have not been fully understood. However, the direct physical continuity between the prostate and the urethra, as the latter is being known as a reservoir for infection by HPV, may possibly explain why HPV DNA has been reported in prostatic tissues. According to this HPV-infected cells shed from the urethra into

seminal fluid likely explain why HPV-DNA has been detected in normal prostatic tissues, benign prostatic hyperplasia, and prostate cancer in some studies [216, 217].

Several small HPV studies of heterosexual partners have been published[218]. In a study comparing HPV status in the cervix and semen of heterosexual partners, Kyo et al. demonstrated that 75% of women whose male partners were HPV positive had HPV DNA in their cervix, while only 39% of the men whose partners were HPV positive carried HPV DNA in their semen[219]. Campion et al. examined HPV disease in women whose sexual partners had penile condyloma, and found that 76% of the women had genital HPV infections, including 36% with abnormal cervical cytology and 27.7% with cervical HPV DNA detected by hybridization [220].

Although few studies define correlates of genital HPV infections in men, consistently positive associations between HPV detection and measures of sexual history, including lifetime and recent number of sexual partners and sexual frequency, have been observed [221]. Conversely, circumcision has been consistently associated with reduced detection of HPV infection in men. In an international study, HPV prevalence was significantly lower among circumcised men [222]. Less consistently, condom use has been associated with reduced risk of HPV detection in men [221]. Most studies found no association between age and genital HPV prevalence in men [218].

1.20. Host response towards HPV infection

There is no viremic phase in the pathogenesis of HPV infection in humans, so the infection is not widely disseminated in the body [176]. Therefore, HPV-associated malignancies would occur at anatomic sites of exposure by direct contact with the virus [223]. In other words, HPV-

caused primary tumours would occur only at sites where the virus can enter the body such as the genital tract, skin, and aerodigestive tract. Tumours of the solid internal organs, such as the brain, the kidney, and the liver, are unlikely to be HPV related [85].

Papillomavirus infection does not usually provoke a prominent immune response. However, HPV infections are in most cases cleared after 18 to 24 months of the exposure [224]. The underlying process of virus clearance is not fully understood yet, but the host immune system in general and the cytotoxic response namely is proposed to be involved [225]. The serum antibodies in humans against HPV are type specific and their levels remain stable over time, after few weeks of infection. Nonetheless, not all the HPV infection individuals will show seroconversion subsequently [226].

Individuals who have been exposed to HPV with or without an associated cancer may have serum antibodies to the viral capsid proteins. These anticapsid antibodies can be detected in many normal individuals because HPV infections are so common [227]. However, serum antibodies to the viral E6 and E7 proteins are strongly associated with invasive cancer and are rarely can be detected in patients without cancer. High levels of E6 and E7 serum antibodies have a greater than 95%–99% specificity and about a 50% sensitivity in invasive cervical cancer cancers [85, 150, 228, 229].

Antibodies to E6 and E7 are not biomarkers for cervical cancer risk and do not play a role in the pathogenesis of disease, but rather they provide specific immunologic evidence for transcription of the viral genome in patients [229]. The specificity of these antibodies to individuals with

invasive cancer is possibly due to presentation of the oncoproteins to the immune system during or after the process of microinvasion [225]. Because of the modest sensitivity of E6 and E7 antibodies, seroreactivity may have limited benefit as a diagnostic method for an individual case [230].

1.21. HPV Infections risk factors

Sexual behaviours associated with increased risk of developing cervical cancer are, in large part, surrogate routes for HPV exposure [231]. It is likely that the transmission of viral infection to non-genital sites occurs in the context of some sexual behaviours, such as oral-genital contact, or by autoinoculation from a genital infection [232]. Sexual behaviours known to be associated with HPV exposure include a high number of lifetime sexual partners, young age at first intercourse, and a history of sexually transmitted disease [233]. Although other mechanisms such as intrapartum transmission as well as transmission through nonsexual or noncoital sexual contact are possible, they are unlikely to be common mechanisms [234]. Interactions between sexual behaviours and other known risk factors for cancers at a particular site must be considered. For example, in the case of head and neck cancers, alcohol consumption and smoking use may confound analysis of sexual behaviour [235, 236].

Malignancies that are causally associated with viral infections would be increased in individuals with immunosuppression [237]. This increase is presumably due to antigen-specific restricted responsiveness in patients with disorders of cell-mediated immunity [238]. All of the tumours with established associations with HPV are known to occur at greater than expected rates in individuals who are immunosuppressed because of HIV or immunosuppression treatment [239, 240].

1.22. Latency of HPV infections

It is estimated that HPV may be harboured in a latent phase for 20 years or longer, before manifesting as a precancerous lesion of the cervix. The latency period between persistent HPV infection and low-grade cervical dysplasia is 5 years on average, and between low-grade lesions and the development of invasive cancer is about 15 years [241]. This relatively long time to progression is partly responsible for the successful reduction in the incidence and mortality statistics for cervical cancers in industrialized nations depending on the cervical smear testing as well as the HPV vaccination for young female adults [242].

Once infected with an oncogenic HPV type, patients with persistent HPV may remain at least intermittently capable of transmitting the disease to sexual partners throughout their lifetime [243].

1.23. Prevention from HPV infections

It has been widely accepted that using condoms can reduce, but do not negate, the risk of infections with HPVs [244]. Moreover, preclinical studies in mouse model suggest that the polysaccharide carrageenan greatly inhibit HPV transmission, whereas the spermicidal compound nonoxynol-9 appears to increase HPV transmission [245].

The first generation prophylactic HPV vaccines consist of recombinant HPV L1 proteins that self-assemble into virus-like particles [246]. Gardasil vaccine has been approved by FDA to vaccinate girls and young women between 9 to 26 years. It is a quadrivalent formulation of the most prevalent low-risk (HPV6 and HPV11) and high-risk HPVs (HPV16 and HPV 18) and it promises to be properly efficient in providing type-specific protection from new infections with these HPV types [247]. Since these prophylactic

vaccines lead to the development of humoral immune response, they are not predicted to affect potential HPV infections at the time of vaccination [246]. It is not clear yet whether other no-vaccine high-risk HPV types will become more prevalent as HPV 16 and HPV18 are to be eradicated from the biological pool [248].

1.24. Hypothesis

It may be hypothesized that infections with high-risk HPVs play a role in the prostate cancer pathogenesis, which might be analogous to that of the cervical cancer, with different incidence among nations or ethnic groups. In addition, the patients who are homozygous for the arginine form at codon 72 of the *p53* gene are more common among prostate cancer patients whose prostate tissues are infected with high-risk HPVs. We attempt to test this hypothesis using a variety of morphologic, molecular, quantitative, and qualitative techniques.

1.25. Aims of the research

The main aim of this research is to examine if infections with HPVs are involved in prostate cancer pathogenesis. In addition, this study aims to find out the genotypes and viral load of any detected HPV infections within the prostate cancer and control groups from different ethnic backgrounds.

Furthermore, this study aims to look for any link between the patients' medical background (PSA tumour marker titration, age), tumour grading, and tumour staging with HPVs infections in the prostate cancer and control groups.

Moreover, this research aims to assess the status of *Rb* and *P53* tumour suppressor genes along with the apoptosis index to evaluate the activity of the high-risk HPVs infections in the study and control groups.

Finally, this research aims to investigate any association between Codon 72 polymorphism of the *p53* gene and high-risk HPV infections in prostate cancer cases.

CHAPTER 2: MATERIALS AND METHODS

2.1. STUDY AND CONTROL SAMPLES

2.1.1. Introduction

In this study, patients with various stages of prostate cancer were selected. These patients were compared with age-matched patients diagnosed with benign prostate hyperplasia conditions.

2.1.2. Case selection

Specimens from the Middle East population (Group I) were obtained from Sheikh Khalifa Medical City and Tawam Hospitals in the United Arab Emirates (UAE). These were histology specimens obtained by TURP, and trans-rectal ultrasound (TRUS) biopsy. Both hospitals treat UAE nationals only. Ethical approvals from the local research ethics committees at both hospitals were obtained formally. Prostate cancer archival tissues were sent to the tissue bank at Middlesex University in London. The other two groups of Caucasians (Group II) and Afro-Caribbean men (Group III) from the East London Region were selected from the prostate cancer database, which were available at the tissue bank at Middlesex University in London. Only those cases with sufficient relevant clinical information were included in the study. Ethical approval was obtained from the natural sciences ethics sub-committee at Middlesex University. The archival tissues from the three groups were then anonymised to avoid any bias during the research course.

2.1.3. The study (prostate cancer) population

Prostate tissue samples from the UK population (n= 74 cases) were subdivided into Caucasians (Group II-PCa, n=43 cases) and Afro-Caribbean men (Group III-PCa, n=31 cases) along with the Middle East population (Group I-PCa, n=49 cases). The first two groups from the UK population were randomly selected from the prostate cancer database over a five-year period between 1997 and 2002. However, because of the low prevalence of prostate cancer in the UAE population, the period was expanded to ten years between 1990 and 2000. All the patients were selected using hospital records, and cancer registry data. Ethnicity of all the cases was determined from the clinical notes, death certificates and patient questionnaires and questionnaires to relatives of deceased patients. Standardized data were extracted from hospital records sent with the paraffin-embedded samples to the tissue bank at Middlesex University. These data covered the stage of the disease at presentation, investigations procedures, biochemical results, treatments, and follow up status of each individual case.

2.1.4. Inclusion criteria in the study group

Patients with prostate cancer from Caucasians, Afro-Caribbean men as well as from the Middle East region were included in the study, from whom archived tissues were available for research at the tissue bank at Middlesex University.

2.1.5. Exclusion criteria from the study group

Six cancer patients were excluded from the groups mentioned above due to lack of relevant clinical information. Finally, only the numbers mentioned above were included in the study.

2.1.6. The control population

Similarly, prostate tissue samples from the three population groups were obtained from the same sources; Middle East (Group I-BPH, n=99 cases), Caucasian (Group II-BPH, n=77 cases) and Afro-Caribbean (Group III-BPH n=91 cases). Cases were selected upon examining the histology specimens obtained by TURP.

2.1.7. Inclusion criteria in the control group

Patients with benign prostatic hyperplasia (BPH) proven by microscopic histopathological examination of TURP specimens of Caucasians, Afro-Caribbean and Middle Eastern patients were included in the study, from whom archived tissues were available for research at the tissue bank at Middlesex University.

2.1.8. Exclusion criteria from the control group

Patients from other ethnic groups and those with insufficient clinical information were excluded.

2.1.9. Age of the patients at presentation

The age distribution of patients ranged between 54 – 90 years (mean=71.5 years) in Group I, between 48 – 88 years (mean=72.2 years) in Group II and between 54 – 90 years (mean=71.5 years) in Group III (Table 2-1).

There was no significant difference between the three groups in terms of age at presentation (Middle East vs. Caucasians $P = 0.3451$, Middle East vs. Afro-Caribbean men $P = 0.4765$, Caucasians vs. Afro-Caribbean men $P = 0.9994$). Therefore, the process of age normalisation of the study and control cases was not required in this study.

Table 2-1: Age distribution in the study and control population groups

| Age groups | Group I PCa | Group I BPH | Group II PCa | Group II BPH | Group III PCa | Group III BPH |
|------------|----------------|----------------|-----------------|-----------------|------------------|------------------|
| 40-49 | 1 | 0 | 0 | 3 | 1 | 8 |
| 50-59 | 3 | 11 | 0 | 8 | 14 | 23 |
| 60-69 | 15 | 42 | 9 | 23 | 12 | 39 |
| 70-79 | 16 | 26 | 25 | 30 | 3 | 13 |
| 80-89 | 10 | 17 | 9 | 13 | 1 | 0 |
| 90-99 | 4 | 3 | 0 | 0 | 0 | 8 |

Group I = Middle East Population.

Group II = Caucasians Population.

Group III = Afro-Caribbean Population.

2.1.10. Prostate cancer cases staging at presentation

Prostate cancer staging have been extracted from the accompanying clinical information of each case in the study subgroup (Table 2-2).

Table 2-2: Prostate cancer staging in the three study subgroups

| Stage (TNM) | | | |
|----------------------------|------------------------------------|----------------------------------------|--------------------------|
| No. of patients and Groups | Organ-confined disease T1-2N0M0 | Locally advanced disease T3-4N0/xM0 | Metastatic T2-4Nx/+M1 |
| Group I | 18 | 8 | 23 |
| Group II | 32 | 5 | 6 |
| Group III | 21 | 7 | 3 |
| Total number of cases | 71 | 20 | 32 |

Group I = Middle East Population.

Group II = Caucasians Population.

Group III = Afro-Caribbean Population.

2.1.11. Serum prostate specific antigen (PSA) values

Serum PSA values were extracted from the clinical history of each individual patient in the study and control groups since presentation and towards the surgical procedure. The average of the PSA value between presentation and the surgical procedure has been taken as the PSA value for each case (Table 2-3).

Table 2-3: The median of PSA value in the study and control

| Patients | PSA (Median µg/L) | | |
|------------|----------------------|----------|-----------|
| | Group I | Group II | Group III |
| PCa | 6.6 | 9.5 | 8.7 |
| BPH | 3.58 | 6.3 | 5.7 |

Group I = Middle East Population.

Group II = Caucasians Population.

Group III = Afro-Caribbean Population.

There was a significant difference in the presenting PSA values in the control subgroups between the Caucasians and the Middle East population, being higher in the former (Middle East vs. Caucasians $P = 0.0116$), while there was no significant difference between the other groups (Middle East vs. Afro-Caribbean men $P = 0.1446$, Caucasians vs. Afro-Caribbean men $P = 0.7849$).

On the other hand, there was a significant difference in the PSA values in the study subgroups between the Afro-Caribbean group, being highest (median: 12.7) and the Caucasians (median: 9.5) and then the Middle East population (median 6.3) ($P = 0.0029$ and $P < 0.0001$ respectively). While there was no significant difference between the latter two groups (II & III) ($P = 0.8865$).

2.1.12. Specimen collection and storage

The archived tissue samples in the form of paraffin blocks were obtained from the tissue bank at Middlesex University.

Samples were anonymised by allocating each sample with a randomly distributed research reference number (identifier). This particular number was then used for the relevant clinical data for each tissue.

2.2. DNA EXTRACTION FROM PARAFFIN-EMBEDDED TISSUES

2.2.1. Introduction

Archival, formalin-fixed, paraffin-embedded tissue is an invaluable resource for molecular genetic studies. Extraction of nucleic acid from archival tissue allows retrospective analysis and correlation of clinical endpoints or histological appearance structures with molecular biological markers.

Three steps are involved in the DNA extraction from paraffin-embedded tissues: deparaffinization this is achieved via dissolution of the wax in xylene and ethanol, digestion of the cellular proteins; this is performed by the use of certain proteinases, and purification of the produced genomic DNA [249, 250].

2.2.2. Materials

The following reagents have been used in all the DNA extractions experiments:

- Chloroform
- EDTA, 0.5 M
- Ethanol, absolute
- Isoamyl alcohol
- Phenol

- Proteinase K
- Sodium acetate, pH 5.2
- TE Buffer (Tris-EDTA), pH 7.4
- Xylene

The following preparation formulas have been used to prepare the working solutions used in the DNA extraction experiments:

A. Chloroform/Isoamyl alcohol 24:1

- Chloroform 24 ml
- Isoamyl alcohol 1 ml

B. DNA extraction buffer

- 1.5 ml 5M NaCL
- 5.0 ml 0.5M EDTA
- 0.5 ml Tween 20
- 93 ml of sterile water

C. Proteinase K (10 mg/ml)

100 mg Proteinase K was dissolved in 10 ml TE for 30 min at room temperature (RT). The stock was divided in to aliquots and stored at -20°.

2.2.3. Method

In Day 1 on the DNA extraction experiments slices (20 µm) of formalin-fixed and paraffin-embedded tumour samples are cut using microtome. Samples are then put in 2 ml *Eppendorf* collection tubes. Tissues were incubated in xylene at 45°C for 15 minutes allowing the wax to dissolve.

Samples were then centrifuged 10 min at 14,000 rpm. Resultant supernatant is pipetted off, leaving the wax in the bottom of the tube. The latter steps were repeated once more. Then 1 ml 100% ethanol added to tissue pellet, vortexed and then centrifuged for 10 min at 14,000 rpm, and supernatant pipetted off. 1 ml 90% ethanol added to tissue pellet, vortexed, centrifuged for 10 min at 14,000 rpm, and supernatant pipetted off.

The same step was repeated, with 70% ethanol this time. The resultant pellet was then dried in speed vac. Pellet is resuspended in 400 μ l of DNA extraction buffer. 40 μ l of Proteinase K (10 mg/ml) was added, vortexed briefly, and incubated at 55°C overnight in order to get rid of the protein.

In Day 2 of DNA extraction experiments 440 μ l of phenol was added, and was shaken vigorously by hand for 5 min, and centrifuged for 5 min at 8000 rpm. Supernatant pipetted into a new tube, a solution of 220 μ l phenol plus 220 μ l chloroform/isoamyl alcohol (24:1) was then added, and shaken vigorously by hand for 5 min, and centrifuged for 5 min at 8000 rpm. The aim was to get rid of the Phenol. Again, the supernatant was pipetted into a new tube, 440 μ l chloroform/isoamyl alcohol (24:1) was added, shaken vigorously by hand for 5 min, and centrifuged for 5 min at 8000 rpm, to get rid of chloroform/isoamyl alcohol. Then, the resultant supernatant pipetted into a new tube (2 ml Eppendorf tube), 1/10 volume of sodium acetate (pH 5.2) added. In addition, 3 volumes of ice-cold 100 % ethanol was added, and tube kept overnight at -20°C.

By day 3 of the DNA extraction experiments the tubes were centrifuged for 30 min at 4°C and the supernatant was removed. The pellet was dried in speed vac. 20-50 μ l sterile water was then added. Tubes were then shaken gently in thermo mixer at 37°C for 2 hours [249, 250].

2.3. ISOLATION OF DNA FROM HeLa CULTURED CELL LINE

2.3.1. Introduction

A HeLa cell line (also Hela or hela cell line) is a cell type in an immortal cell line used in scientific research. It is one of the oldest and most commonly used human cell lines. The line was derived from cervical cancer cells taken on February 8, 1951 from Henrietta Lacks, a patient who eventually died of her cancer on October 4, 1951. The cell line was found to be positive for HPV18 [251].

2.3.2. Materials

- HeLa cell line sample in a culture flask.
- Lyophilized QIAGEN protease (QIAGEN).
- 50 ml FlexiGene DNA Kit (QIAGEN).
- Isopropanol (100%).
- Water bath.
- Centrifuge.
- 70% ethanol.
- Sterile 0.5 ml *Eppendorf* tubes (Fisher).

2.3.3. Method

HeLa cells were grown in a monolayer and could be harvested by detaching the cells from the culture flask using a cell scraper. By using a cell scraper, the appropriate number of cells was transferred to a 1.5 ml microcentrifuge tube and centrifuged for 5 min at 300xg. The supernatant was removed completely with extra care not to disturb the pellet and

discard. 300µl buffer FG1 were added to the cell pellet and mixed by pipetting up and down until the cells were resuspended. 300µl Buffer FG2/QIAGEN Protease were added, and then the tube was closed and inverted few times. Afterwards, the tube was put in the water bath, and incubated at 65°C for 10 min. At this stage 600 µl isopropanol (100%) were added and mixed thoroughly by inversion until the DNA precipitate becomes visible as threads or a clump. Complete mixing with isopropanol is so vital to precipitate the DNA and must be checked by inspection. Then the tube was centrifuged for 3 min at 10,000xg, but if the resulting pellets were loose, centrifugation could be prolonged or a higher g-force could be used.

The supernatant was discarded, and briefly, the tube was inverted onto a clean piece of absorbent paper, taking care that the pellet remains in the tube. Then 600µl 70% ethanol were added and vortexed for 5 seconds, followed by centrifuging for 3 min at 10,000 x g, but if the resulting pellet was loose, centrifugation could be prolonged or a higher g-force could be used.

The supernatant was discarded and the tube was left inverted on a clean piece of absorbent paper for at least 5 min, with extra care that the pellet remained in the tube. In rare cases, the pellet may be loose, so pouring was done slowly and with extra care not to lose the pellet. Inverting the tube onto absorbent paper minimized backflow of ethanol from the rim and sides of the tube onto the pellet.

The DNA pellet was air-dried until all the liquid was evaporated for at least 5 min but without over-drying the DNA pellet, since over-dried DNA is very difficult to dissolve. 200 µl buffer FG3 was added then to the tube, vortexed for 5 s at low speed. The DNA was dissolved by incubating for 30 min at 65° C in a water bath. Nevertheless, if the DNA was not completely dissolved, the incubation was prolonged until the DNA is

completely dissolved. With some cell samples, traces of the pellet that do not contain DNA may remain undissolved after 1 h incubation at 65°C. They could be removed by centrifuging for 1 min at 10,000xg, and transferring the supernatant into a fresh tube. If a reduced volume of Buffer FG3 was used, incubation time was to be prolonged.

2.4. DNA QUANTIFICATION AND NORMALISATION

2.4.1. Introduction

The Spectrophotometer is a useful tool in the laboratory for determining the quality and quantity of the DNA samples. The sample can be analyzed by passing various sized ultraviolet (UV) wavelengths through it. DNA samples are commonly scanned at wavelengths of 260 nm, 280 nm, 320 nm, and 234 nm. DNA absorbs UV light at a maximum of 260 nm and a minimum of 234 nm. Absorbance at 320 nm may indicate a dirty cuvette or particulates in the sample [252].

2.4.2. Materials

- UV-3600 UV-Vis-NIR Spectrophotometer (Shimadzu).
- DNA samples.
- Microcentrifuge tubes.
- Sterile water.
- TE buffer.
- Quartz Cuvette for UV-3600 UV-Vis-NIR Spectrophotometer (Shimadzu).

- Kimberly Clarke wipes.

2.4.3. Method

The quartz cuvette of spectrophotometer required at least 100 μ l of liquid to cover the windows. Smooth sides of the cuvette were not touched at all times as any smudges on the windows could result in distorted readings. The cuvette was handled on the frosted sides only. To clean the quartz cuvette 100 μ l of distilled water were added to the cuvette, and then the cuvette was emptied into a liquid waste beaker and carefully shaken to empty all water droplets from it, and then dried from the outside and the smooth sides with a Kimberly Clarke wipe.

The first step to set up the Shimadzu of the spectrophotometer software to read and record wavelengths was clicking on the Shimadzu icon on the computer screen connected to the spectrophotometer, then the system was zeroed prepared by clicking on the "Zero" button on the left side of the screen with the sample compartment empty and the door closed. Then "Setup" was clicked on the left side of the screen, then "Read at Wavelength" was chosen, and from the active drop menu, the desired wavelength was selected. Finally, Ok was clicked when the set up was completed.

In a properly sized tube (*i.e. microcentrifuge tube or 1.5 ml tube*), the desired amount of the sample to be analysed was added. The desired dilution was obtained by adding the needed amount of TE buffer. With the cap of the tube on, the content was mix with a vortex for up to 1 minute. The spectrophotometer was zeroed then by pressing the "Zero" button to the left of the screen while the sample compartment is empty and the door is closed. After that 200 μ l of the prepared sample were added to a quartz cuvette.

Then, the quartz cuvette was placed in the square, black sample compartment in a way that the frosted sides will be facing the front and back, and the small windows will be facing the left and right. The spectrophotometer door was closed then and the “Read” icon on the software was clicked to obtain the reading wavelength value for the parameters set up. The same reading wavelengths procedure for any remaining samples were repeated for all the samples to be tested, so each subsequent sample will be added to the current page, making a list of data on the software.

The following calculation formulas have been used:

Concentration Formulas

| Determining Concentration |
|--------------------------------------------------------------------------------------------------------------|
| $A_{260} \times 50\mu\text{l/ml} \times \text{dilution factor} = \text{DNA concentration}$ |
| $A_{260} \times 40\mu\text{l/ml} \times \text{dilution factor} = \text{RNA concentration}$ |

Purity Formulas

| Determining Concentration |
|------------------------------------------------------------------------------------------------------|
| $A_{260}:A_{280} = A_{260} / A_{280}$ |
| $A_{234}:A_{260} = A_{234} / A_{260}$ |
| $A_{320} / A_{226} \times 100 = \% \text{ UV absorbed by particles/dirty cuvette}$ |

Since both DNA absorb UV light maximally at 260 nm, the A₂₆₀ value generated from the simple reads report is used. When the concentration of DNA is 50μl/ml, the absorbance reading is 1.0. When the concentration of RNA or single-stranded DNA is 40μl/ml, the absorbance reading is 1.0. To determine the concentration of the sample the already mentioned formula was used.

Calculating ratios such as the A260:A280 ratio and the A234:A260 ratio can be used to determine the purity of a sample of DNA. The A260:A280 ratio of DNA indicates protein or RNA contamination. This value should be between 1.8 and 1.9. A high A260:A280 ratio may be caused by RNA contamination while a lower ratio can be caused by protein contamination.

The A234:A260 ratio is used to determine the presence of protein or phenol contamination. A sample contaminated with protein or phenol has a ratio greater than 0.50. The absorbency values can be found on the Simple Reads Report generated from the above procedure. To determine the above ratios the already mentioned formula for calculating the purity was used.

The A320 reading was used to determine the presence of particulates in the sample or a dirty cuvette. If either exists, the reading may be skewed. The A320 value should be less than 5% of the A260 value. The already mentioned purity calculation formula was used to ensure readings are accurate. The final genomic DNA concentration has been normalised in all the study and control samples to be 100 ng/25 µl final PCR reaction. This could be done by diluting the extracted DNA if the DNA concentration was found over 100 ng/µl. When the DNA concentration found to be less than 100 ng/µl, the starting DNA normalisation could be applied by adding sufficient amount of DNA sample to the PCR reaction to maintain the required concentration of the starting DNA in the PCR reaction. In the latter cases, the amount of the PCR graded water have been corrected before being added to the final PCR reaction in line with the additional amount of DNA samples added to the reaction.

2.5. POLYMERASE CHAIN REACTION (PCR)

2.5.1. Introduction

PCR is a method for amplifying a DNA base sequence using a heat-stable polymerase and two specific primers, one complementary to the (+ve)-strand at one end of the sequence to be amplified and the other complementary to the (-ve)-strand at the other end. Because the newly synthesized DNA strands can subsequently serve as additional templates for the same primer sequences, successive rounds of primer annealing, strand elongation and dissociation produce rapid and highly specific amplification of the desired sequence.

PCR, as currently practiced, requires several basic components. These components are as follows; DNA template, which contains the region of the DNA fragment to be amplified, in addition to two primers, which determine the beginning and end of the region to be amplified. Also, Taq polymerase (or another durable polymerase), which is DNA polymerase is required. This polymerase copies the region to be amplified. Moreover, deoxynucleotides-triphosphate is needed, from which the DNA polymerase builds the new DNA. Finally, a buffer is required, which provides a suitable chemical environment for the DNA polymerase.

The PCR process is carried out in a thermal cycler, which is a machine that heats and cools the reaction tubes within it to the precise temperature required for each step of the reaction. To prevent evaporation of the reaction mixture (typically volumes between 15-100 μ l per tube), a heated lid is placed on top of the reaction tubes or a layer of oil is put on the surface of the reaction mixture.

The DNA fragment to be amplified is determined by selecting primers. Primers are short, artificial DNA strands — often not more than 50 and usually only 18 to 25 base pairs long — that are complementary to the

beginning or the end of the DNA fragment to be amplified. They anneal by adhering to the DNA template at these starting and ending points, where the DNA polymerase binds and begins the synthesis of the new DNA strand.

The choice of the length of the primers and their melting temperature (T_m) depends on a number of considerations. The melting temperature of a primer - not to be confused with the melting temperature of the template DNA - is defined as the temperature at which half of the primer binding sites are occupied. Primers, which are too short, would anneal at several positions on a long DNA template. This would result in non-specific copies. On the other hand, the length of a primer is limited by the maximum temperature allowed to be applied in order to melt it, as melting temperature increases with the length of the primer. Melting temperatures that are too high, i.e., above 80°C, can cause problems since the DNA polymerase is less active at such temperatures. The optimum length of a primer is generally from 15 to 40 nucleotides with a melting temperature between 55°C and 65°C [253].

2.5.2. Materials

- Techne 3 Prime Thermal Cyclor (Techne).
- Sterile PCR tubes (ABgene).

The PCR mix has been prepared as follows:

- 2.0µl of DNA.
- 2.5µl of MgCl₂ (25mM).
- 2.5µl of 10X buffer (75mM Tris-HCl pH 8.8, 20mM (NH₄)₂SO₄).
- 0.3µl of dNTPs (300µM of each of the dNTPs: dATP, dGTP, dCTP, dTTP) (ABgene).

- 2.0µl of primers (forward and reverse: 2.5 pmol each)
- 0.2µl of *Taq* polymerase 5 units/µl (ABgene)
- 15.5µl of H₂O

2.5.3. Method

In this research three primer sets have been used to detect the HPV infections in the extracted genomic DNA (Table 2-4); GP5+/GP6+ consensus primers [254], and MY09/11 degenerate primers [255] , in addition to broad-spectrum multiplex PCR primer set SPF1/2 [256]. These three PCR assays were designed to amplify distinct highly conserved L1 ORF regions of the HPV genome with different amplicon sizes (Figure 2-1) [256].

The samples were screened for the presence of HPV using the standard nested PCR approach, consisting of the MY09/11 degenerate primer set as outer primers and the GP5+/GP6+ consensus primer set as inner primers. This nested PCR method has been collectively called MY/GP [257].

The Degenerate primers may be used to amplify DNA in situations where only the protein sequence of a gene is known, or where the aim is to isolate similar genes from a variety of species. A six or seven residue peptide sequence should be selected, corresponding to an oligo of about 20 nucleotides. If the oligo is designed to amplify several similar protein sequences, then the most conserved regions of the proteins need to be selected. If some of the residues are not completely conserved, then the oligo sequence will need to accommodate all possible codons of all amino acid residues at that site. The peptide sequence should avoid amino acids that have a lot of codons, such as leucine (L), arginine (R), and serine (S). Instead, aim for regions that are rich in amino acids that have only one or

two possible codons (i.e. M, W, C, D, E, F, H, K, N, Q, Y). Inosine residues can pair with any nucleotide, and so can be used at sites where there is complete degeneracy. This reduces the number of oligos that have to be synthesised [258, 259].

Table 2-4: The design of MY 09/11 and GP5+/GP6+ primer sets, as well as SPF1/2 cocktail of six primers .

| Primer Designations | Sequence 5' → 3' | Amplimer Size |
|---------------------|---------------------------------|---------------|
| MY09* | 5'-CGTCCMARRGGAWACTGATC-3' | 450 bp |
| MY11* | 5'-GCMCAGGGWCATAAAYAATGG-3' | 450 bp |
| GP5+ | 5'-TTTGTACTGTGGTAGATACTAC-3' | 150 bp |
| GP6+ | 5'-GAAAAATAAACTGTAAATCATATTC-3' | 150 bp |
| SPF1A | 5'-GCiCAGGGiCACAATAATGG-3' | 65 bp |
| SPF1B | 5'-GCiCAGGGiCATAACAATGG-3' | 65 bp |
| SPF1C | 5'-GCiCAGGGiCATAATAATGG-3' | 65 bp |
| SPF1D | 5'-GCiCAAGGiCATAATAATGG-3' | 65 bp |
| SPF2B-bio** | 5'-GTiGTATCiACAACAGTAACAAA-3' | 65 bp |
| SPF2D-bio** | 5'-GTiGTATCiACTACAGTAACAAA-3' | 65 bp |

*Degenerate primers MixBase codes definition

R= A, G

Y= C, T

M= A, C

W= A, T

** bio= biotinylated

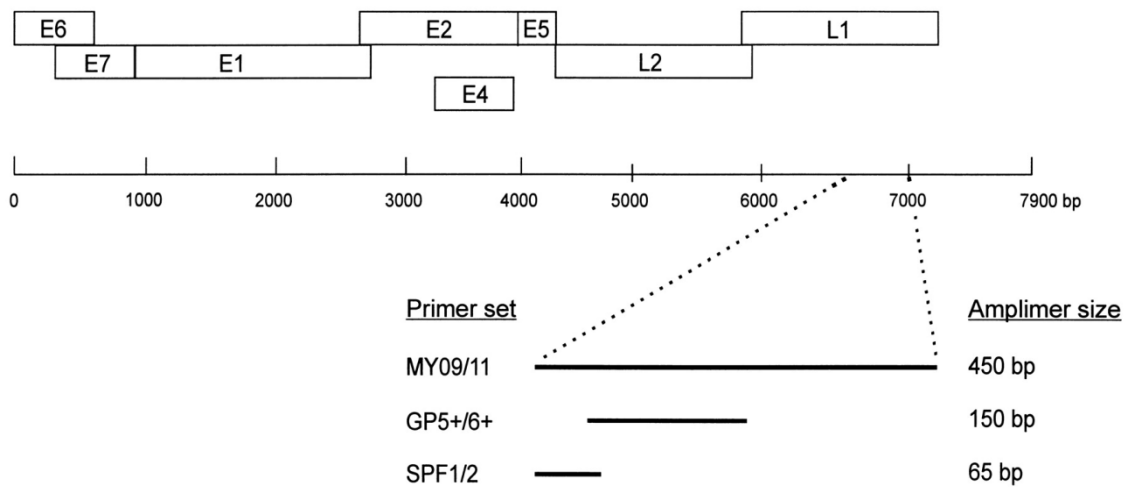


Figure 2-1: Primer targets of the HPV primer sets and their amplicon size .

Housekeeping genes primers were selected to check the integrity of DNA as internal control. So to exclude false-negative results, the adequacy of each DNA sample was confirmed before any PCR assay to detect HPV DNA in each case by β -globin (110 bp) PCR assay using PCO3 primer 5'-ACACAACTGTGTTCACTAGC-3' and PCO4 primer 5'-CAACTTCATCCACGTTTACC-3'.

All DNA samples extracted from the paraffin embedded tissues of study and control groups have been tested by PCR with each of the three sets of primers, in addition to the nested PCR method, separately.

Positive and negative controls have been included in all the experiments. Extracted genomic DNA preparations from HeLa cell lines (containing HPV18) were used as positive controls. PCR grade deionized purified water was used instead of the DNA template as a negative control in each experiment. The starting DNA concentrations have been normalised among to be 100ng/ μ l in all the samples before all the PCR assays using

standard spectrophotometry and dilution method with PCR grade deionized purified water.

For a 25 μ l PCR run the following amounts were used: 1 μ l of 100ng/ μ l DNA in addition to 1 μ l of each primer at 3.2 pmole/ μ l concentration or 1.25 μ l of each primer at 100ng/ μ l concentration. Also, 2.5 μ l 10x PCR buffer (1.5mM) plus 0.5 μ l 25mM MgCl₂, 0.5 μ l dNTP and 0.125 μ l of 5 unit/ μ l Taq. Finally, 18.37 μ l sterile water to equal a 25 μ l run was added.

All the reagents were kept on ice, and the Taq was added at last, and was kept in the freezer until it was needed during the course of the preparation of the PCR experiment. The content of the PCR test tube was vortexed briefly and quickly spinned before starting the PCR machine.

The optimised PCR conditions using MY09/11 degenerate primer set were 5 minutes at 94°C for initial denaturation, 40 cycles of denaturation at 94°C for 1 minute, annealing at 54°C for 1 minute, elongation at 72°C for 1 minute, and a final elongation step at 72°C for 6 minutes. In addition, GP5+/GP6+ primer set optimised protocol was 40 cycles included a 1 minute denaturation step at 94°C, an annealing step to 40°C for 2 minutes and a chain elongation step to 72°C for 5 minutes. The first cycle was preceded by a 4 minutes denaturation at 94°C and the last cycle was extended by a 4 minutes elongation at 72°C. Finally, the optimised conditions for multiplex PCR using SPF1/2 cocktail of primers were preheating step for 3 minutes at 94°C, which was followed by 40 cycles of 1 minute denaturation step at 94°C, 1 minute for the annealing step at 45°C, and a chain elongation step for 1 minute at 72°C and a final extension of 5 minutes at 72°C.

2.6. AGAROSE GEL ELECTROPHORESIS

2.6.1. Introduction

Gel electrophoresis is a widely used technique for the analysis of nucleic acids and proteins. Most every molecular biology research laboratory routinely uses agarose gel electrophoresis for the preparation and analysis of DNA. Agarose gel electrophoresis was used in this research to determine the presence and size of PCR products.

Electrophoresis is a method of separating substances based on the rate of movement while under the influence of an electric field. Agarose is a polysaccharide purified from seaweed. An agarose gel is created by suspending dry agarose in a buffer solution, boiling until the solution becomes clear, and then pouring it into a casting tray and allowing it to cool. The result is a flexible gelatin-like slab. During electrophoresis, the gel is submersed in a chamber containing a buffer solution and a positive and negative electrode. The DNA to be analyzed is forced through the pores of the gel by the electrical current. Under an electrical field, DNA will move to the positive electrode and away from the negative electrode because of the natural negative charge of the genomic DNA.

Several factors influence how fast the DNA moves, including; the strength of the electrical field, the concentration of agarose in the gel and most importantly, the size of the DNA molecules. Smaller DNA molecules move through the agarose faster than larger molecules. DNA itself is not visible within an agarose gel. The DNA will be visualized by the use of the loading dye that binds to DNA.

The purpose of the gel electrophoresis experiment is to determine the presence or absence of PCR products and quantify the size of the DNA molecule in the PCR product.

TBE or Tris/Borate/EDTA, is a buffer solution containing a mixture of Tris base, boric acid and EDTA. In molecular biology, TBE buffer is often used in procedures involving nucleic acids including electrophoresis. Tris-acid solutions are effective buffers for slightly basic conditions, which keep DNA deprotonated and soluble in water. EDTA is a chelator of divalent cations, particularly of magnesium (Mg^{2+}). As these ions are necessary co-factors for many enzymes, including contaminant nucleases, the role of the EDTA is to protect the nucleic acids against enzymatic degradation. But since Mg^{2+} is also a co-factor for many useful DNA-modifying enzymes such as restriction enzymes and DNA polymerases, its concentration in TBE buffers is generally kept low (typically at around 1 mM).

2.6.2. Materials

The following materials and reagents have been used in the gel electrophoresis experiments:

- Agarose powder (Sigma)
- 10 X TBE buffer
- Gel casting tray with combs (BioRed)
- Gel electrophoresis chamber (BioRed)
- Gel electrophoresis power supply (BioRed)
- Water proof paper based tape
- 500 ml flask
- Sterile water
- 850 watt domestic microwave
- Ethidium bromide (Sigma)
- 6X sample loading dye (Promega)

- 500bp DNA ladder (Promega)
- Transilluminator ultraviolet light-box (Techne)
- UV protective eyewear (Techne)

2.6.3. Method

The following formula has been used to prepare the TBE buffer:

| Component | Amount | 10X Stock Concentration | Final Concentration |
|---------------|--------|----------------------------|------------------------|
| Tris Base | 108 g | 890 mM | 89 mM |
| Boric Acid | 55 g | 890 mM | 89 mM |
| EDTA (pH 8.0) | 40 ml | 20 mM | 2 mM |

Usually for convenience, a concentrated stock of TBE buffer (10X) is made ahead of time and diluted with distilled water to 1X concentration prior to use.

For preparing the 1% agarose gel; 2 g agarose powder was measured and added to a 500 ml clean flask, and then 200 ml TBE buffer was added to the flask. The total gel volume will vary depending on the size of the casting tray used at the day of experiment. The agarose powder was melted then in a microwave until the solution becomes clear by heating the solution for several short intervals to avoid boiling the solution for long periods as it may boil out then of the flask. After that, the solution was left to cool down to about 50-55°C, by swirling the flask occasionally to cool evenly. At this stage 10µl of Ethidium Bromide were added to the agarose solution.

The ends of the casting tray were sealed with two layers of special water proof paper based tape and the combs were put in the gel-casting tray, then the melted agarose solution was poured into the casting tray and left to cool until it is solid for about 30-35 minutes.

At this stage, the combs were carefully pulled out and the sealing tapes were removed, and the gel moved to the electrophoresis chamber.

Enough TBE Buffer was added so that there is about 2-3 mm of buffer over the gel.

Gels could be made several days prior to use and sealed in plastic wrap (without combs). If the gel became excessively dry, we could allow it to rehydrate in the buffer within the gel box for a few minutes prior to loading samples.

6 μ l of 6X sample loading dye were added to each of the 25 μ l PCR reaction. The order of each sample was loaded on the gel was recorded on the lab book, including the DNA template identifier number in addition to controls and ladder. At this stage, 20 μ l of each sample/sample loading dye mixture were pipetted carefully into separate wells in the gel. Also, 10 μ l of the DNA ladder were pipetted into at least one well of each row on the gel, which is usually the first one on the left hand side.

Once all the samples are loaded, the gel apparatus can be covered by its translucent lid. The leads of the gel apparatus can be connected then so that the red (positive) lead is at the end of the gel to which the DNA will migrate and the black (negative) lead is at the end of the gel containing the wells. At this stage, the power supply can be turned on to run the gel at a constant voltage of 200 volts. When the blue tracking dye (which runs in these gels along with a DNA fragment of about 65-450 bp) has migrated about 75% of the distance to the end of the gel (usually within 60-90 minutes), the power supply can be turned off then and the power leads can be disconnected afterwards.

Then the DNA samples can be visualized with UV light-box on the Lumi-Imager software. The gel should be disposed after the experiment in the clinical waste bin.

In this research, the PCR products were electrophoresed in 1.5%, 2%, 2%, and 4% agarose gels and stained with ethidium bromide for the PCR products of MY09/11, GP5+/GP6+, MY/GP and SPF1/2 PCR assays respectively. The agarose gel concentrations had been optimised using positive control samples with each PCR assay product separately.

2.7. HPV DNA QUANTIFICATION

2.7.1. Introduction

The MultiNA microchip electrophoresis system quickly and easily performs DNA and RNA nucleic acid size confirmation and quantification. This system uses microchip technology to conduct fully automated high-speed electrophoresis separation, and fluorescence detection to perform high-sensitivity analysis.

2.7.2. Materials

The following materials and reagents have been used for the DNA quantification of the multiplex PCR product using SPF1/2 primer sets:

- MCE-202 MultiNA device with DNA-500 on-chip mixing mode (Shimadzu).
- DNA-500 Reagent Kit for MultiNA (Shimadzu) Cat No: P/N 292-27910-91.

- SYBR[®]Gold nucleic acid gel florescent dye manufactured by Invitrogen Cat No : S-11494
- 25 bp DNA ladder (20ng/ μ L) manufactured by Invitrogen Cat No: 10597-011.

2.7.3. Method

The first step for quantifying the HPV DNA concentration in multiplex PCR product using SPF1/2 primer sets was to return MultiNA proprietary reagents (separation buffer, markers) and fluorescent dye reagent to room temperature. Then the analysis schedule was created by starting the PC-based MultiNA instrument control software. A new analysis schedule was registered on the MultiNA instrument control software according to the number of tested samples.

The MultiNA reagents were prepared at this stage; firstly the separation buffer were prepared by diluting the fluorescent dye and stirring it for 2 minutes, and also the specified quantity of marker were prepared according to the mixture mode, and finally the required quantity of ladder for generating the size calibration curve were prepared.

The instrument was loaded before the analysis is being conducted for the first time, and the microchip(s) were set in the instrument. The ladder was set then, sample, separation buffer, and marker at the position registered in the analysis schedule. The wash water was replenished and the waste liquid bottle was emptied.

The samples analysis started after closing the top cover, and pressing the start button displayed in the MultiNA instrument control software. To interpret the results the view data file button was pressed in the MultiNA instrument control software. The viewer opened then to allow viewing of the results for samples already analysed, and give quantification of the

DNA concentration in the sample when each sample analysis page was opened.

2.8. HPV GENOTYPING

2.8.1. Introduction

The reverse hybridization line probe assay technology is based on the reverse hybridization principle, in which part of the L1 region of the HPVs genome is amplified and denatured biotinylated amplicons are hybridised with specific oligonucleotide probe immobilised on a test strip. After hybridisation and stringent washing, streptavidin-conjugated alkaline phosphatase should be added to bind to any biotinylated hybrid previously formed. Thereafter, incubation with BCIP/NBT chromogen would give a purple/brown precipitate. The results can be interpreted then visually by looking for certain colour band according to the subtype of the HPV infection involved in the sample [260]. This technology is able to differentiate between high risk, low risk, and mixed HPV infections found in the samples and controls which showed positivity for HPV DNA using PCR.

2.8.2. Materials

- INNO-LiPA HPV Genotyping Extra Kit (Innogenetics).
- Distilled or deionized water.
- Disposable gloves.
- Disposable DNA/Dnase-free pipette tips (aerosol resistant).
- Forceps for strip handling.
- Graduated cylinders (10, 25, 50, and 100 ml).

- Adjustable pipettes to deliver 1 - 20 μ l, 20 - 200 μ l, and 200 - 1000 μ l.
- Vortex mixer or equivalent.
- Microcentrifuge.
- Water bath (temperature adjustable to $49^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$).
- Aspiration apparatus.
- Calibrated thermometer.

2.8.3. Method

In this research, 10 μ l of multiplex PCR product amplified, containing biotin moieties at the 5' ends of the SPF1/2 primers were denatured by adding 10 μ l of NaOH solution. After 10 min, an INNO-LiPA strip was put into the tray. Two millilitres of prewarmed (37°C) hybridization buffer $3\times$ SSC (45 mM Na-Citrate and 450 mM NaCl, 0.3% Sodium Dodecyl Sulfate) was added and incubated at $50 \pm 0.5^{\circ}\text{C}$ for 1 h. The strips were washed twice for 30 s and once for 30 min at 50°C with 2 ml of hybridization solution. Following this stringent wash, the strips were incubated with 2 ml of alkaline phosphatase-streptavidin conjugate for 30 minutes at room temperature. Strips were washed twice with 2 ml of rinse solution and once with 2 ml of substrate buffer. Two millilitres of substrate (5-bromo-4-chloro-3-indolylphosphate and nitroblue tetrazolium) was added and incubated for 30 min at room temperature. The reaction was stopped by aspiration of the substrate solution and addition of 2 ml of distilled water. After drying, the strip results were interpreted by eye by comparing the hybridization pattern to standard type-specific templates.

The HPV type was determined by eye according to the following criteria. In most cases, the probe name is directly linked to the HPV type (e.g., a purple colour on probe line 16 indicates the presence of HPV-16). Probes c31, c56, and c68 are secondary probes, which were of interest only when

there is a positive hybridization with the probe line just above (31/40/58, 56/74, or 68/45). These c probes were developed in order to discriminate between infections with single HPV genotypes and mixed infections. A true infection with HPV-31 yields positive hybridization with probe line 31/40/58 as well as c31. The c probes c31, c56, and c68 will also react with other HPV types, which have completely matching sequences. Probe c31 also reacts with amplimers from types 33 and 54.

Since the INNO-LiPA does not contain a separate probe for HPV-54, a sample containing type 54 will react exclusively with probe c31. Similarly, amplimers of type 58 will show reactivity with probes 30/40/58, c56, and 58. Probe c68 is also reactive with amplimers from types 68 and 39. HPV-74 is identified by the probes 56/74 and 74. In summary, HPV genotypes 6, 11, 16, 18, 34, 35, 39, 42 to 44, 51 to 54, 59, 66, and 70 are recognized by hybridization to a single probe line, whereas HPV types 31, 33, 39, 40, 45, 56, 58, 68, and 74 yield a specific hybridization pattern on the INNO-LiPA system.

2.9. *p53* GENE CODON 72 POLYMORPHISM

2.9.1. Introduction

Several naturally occurring polymorphisms are found within *p53* and one at codon 72, a C-G transition, alters *p53* 3D structure. At codon 72, either CCC encodes proline (Pro) or CGC encodes arginine (Arg). It has been shown that the E6 proteins from high-risk HPVs are able to target *p53* protein when the *p53* gene codon 72 is homozygous for Arg more efficiently than *p53* Pro to induce a ubiquitin-mediated degradation of the *p53* protein [261]. Consistent with this observation, the majority of HPV-associated tumors so far analyzed are homozygous for the *p53* Arg allele,

whereas the majority of the comparable normal population are heterozygous [262].

2.9.3. Materials

- Techne 3 Prime Thermal Cycler (Techne).
- Sterile PCR tubes (ABgene).

The PCR mix has been prepared as follows:

- 2.0µl of DNA.
- 2.5µl of MgCl₂ (25mM).
- 2.5µl of 10X buffer (75mM Tris-HCl pH 8.8, 20mM (NH₄)₂SO₄).
- 0.3µl of dNTPs (300µM of each of the dNTPs: dATP, dGTP, dCTP, dTTP) (ABgene).
- 2.0µl of primers (forward and reverse: 2.5 pmol each)
- 0.2µl of *Taq* polymerase 5 units/µl (ABgene)
- 15.5µl of H₂O
- For the purpose of this experiment, four PCR primers have been designed and synthesised to determine whether *p53* codon 72 polymorphism plays a role in prostate cancer pathogenesis[263]. Primer pairs were used for the amplification. For the Arginine allele *p53*⁺/Arg⁻ of PCR product of 141 bp; for the Proline allele pro⁺/*P53*⁻ gives a 177 bp product (Table 2-5).

Table 2-5: List of the primers used to study P53 codon 72 polymorphism.

| Primer | Sequence | Length |
|--------|-------------------------|--------|
| P53+ | TCC CCC TTG CCG TCC CAA | 141 |
| Arg - | CTG CTG CAG GGG CCA CGC | |
| Pro + | GCC AGA GGG TGC TCC CCC | 177 |
| P53 - | CGT GCA AGT CAC AGA CTT | |

2.9.3. Method

The *p53* gene codon 72 polymorphism detection technique is based on differences in fragment size for the two forms of the codon 72 of the *p53* gene, which are the *p53*-Pro allele that yields a 177-bp fragment by PCR assay as well as the *p53*-Arg allele, which yields a 141-bp fragment by allele-specific PCR [264].

There possible PCR assay outcomes were: 1) if a PCR product (141 bp) was obtained only with the arginine-specific primers, the patient was considered arginine homozygous, 2) if only a proline-specific primer product (177 bp) was obtained, the patient was considered proline homozygous, 3) if the sample showed amplification with both two primer sets, the patient was considered heterozygous (Arg/Pro) (Figure 2-2)[265].

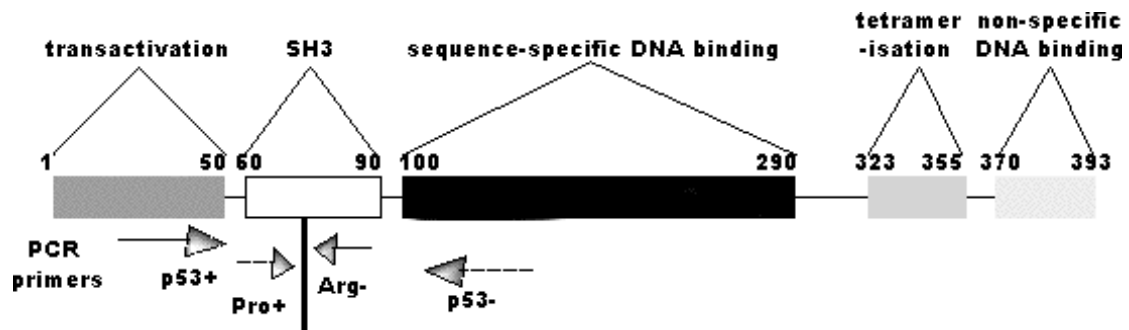


Figure 2-2: The functional domains of the P53 gene, the location of the polymorphism at codon 72, and the position of the primers used for PCR (indicated by arrows).

The PCR optimised conditions were as follows for the *p53*-Pro72 allele: denaturation at 94°C for 5 minutes, then 35 cycles with denaturation at 94°C for 1 minute, annealing at 56°C for 1 minutes and elongation at 72°C for 1 minute. In the last cycle, the elongation step was extended to 10 minutes. For the *p53*-Arg72 allele, the PCR conditions were identical with the exception that annealing was performed at 62°C for 1 minute. The PCR products were separated in 3 % agarose gels.

2.10. HAEMATOXYLIN AND EOSIN (H&E)

2.10.1. Introduction

H&E is the primary conventional stain for every histological specimen study under light microscope. Haematoxylin is a basic histological stain that stains basophilic structures in the cell blue. The basophilic structures are usually those containing nucleic acid like; ribosomes, nucleus, and cytoplasmic regions full of RNA.

Eosin is alcohol based stain with acidic PH, which colours eosinophilic structures bright pink. The eosinophilic structures are generally composed of intra and extra cellular proteins. The Eosin is used for full cellular details enhancement.

2.2.2. Materials

The following materials were required for H&E staining: slides glass rack, staining jar, xylene, absolute alcohol, alcohol 90%, alcohol 70%, distilled water, Giles haematoxylin, acid alcohol, eosin Y solution, 1% acid alcohol solution , xylene based mounting medium, glass slides cover slips.

2.10.3. Method

5 μm sections were cut from the paraffin blocks using standard microtome, then the sections were put in a 40^o C water bath and picked up using glass slides and left to dry overnight.

The slides were deparaffinised in 2 changes of xylene for 5 minutes each, then re-hydrated in 2 changes of absolute alcohol for 3 minutes each. The re-hydration continued in 90% alcohol for 3 minutes and 70% alcohol for another 3 minutes. The slides were washed briefly in distilled water and stained in Haematoxylin solution for 6 minutes. Differentiation of the sections was carried out in 1% acid alcohol for 20 seconds, and then the slides left under running water for 5 minutes. The slides were then counterstained in eosin Y solution for 30 seconds to 1 minute.

Thereafter, dehydration through 70% alcohol was completed for 1 minute, followed by 90% alcohol for 3 minutes and finally 2 changes of absolute alcohol for 3 minutes each. The slides were cleared in 2 changes of xylene for 5 minutes each. Afterwards the slides were mounted with xylene based mounting medium and allowed to dry overnight. On the following

working day, the slides were cleaned using surgical knife and xylene to take out any excess of mounting media and then examined under the light microscope.

2.11. TUMOUR GRADING BY GLEASON SYSTEM

2.11.1. Introduction

Although numerous grading systems exist for the evaluation of prostatic adenocarcinoma, the Gleason grading system is the most widely accepted [266]. The Gleason system is based on the glandular pattern of the tumour as identified at relatively low magnification [267]. Cytological features play no role in the grade of the tumour [268]. Both the primary (predominant) and the secondary (second most prevalent) architectural patterns are identified and assigned a grade from 1 to 5, with 1 being the most differentiated and 5 being the least differentiated [269].

2.11.2. Method

In Gleason pattern 1 and pattern 2, the tumorous tissues are composed of relatively circumscribed nodules of uniform, single, separate, closely packed, medium-sized glands. While, in Gleason pattern 3 the malignant tissues infiltrates the non-neoplastic prostatic tissue, and the glands have marked variation in size and shape, with smaller glands than seen in Gleason pattern 1 or 2. In Gleason pattern 4 the cancerous glands are no longer single and separate as seen in patterns 1 to 3 and large, irregular, cribriform glands can be seen in contrast with the smoothly circumscribed smaller nodules of cribriform Gleason pattern 3. Tumours with this pattern have a significantly worse prognosis than those with pure Gleason pattern 3

[270]. Gleason pattern 5 tumour shows no glandular differentiation and is composed of solid sheets, cords, single cells, or solid nests of tumour with central comedonecrosis [271].

2.12. IMMUNOHISTOCHEMISTRY

2.12.1. Introduction

Immunohistochemistry (IHC) is a method for demonstrating the presence and location of proteins in tissue sections. Immunohistochemical staining is accomplished with antibodies that recognize the target protein. Since antibodies are highly specific, the antibody will bind only to the protein of interest in the tissue section. The antibody-antigen interaction is then visualized using either chromogenic detection, in which an enzyme conjugated to the antibody cleaves a substrate to produce a coloured precipitate at the location of the protein.

To achieve a stronger IHC signal avidin-biotin complex (ABC) can be used. This technique utilizes the high affinity of avidin, a protein found in chicken egg white, for biotin, an enzyme co-factor in carboxylation reactions. Avidin has four binding sites for biotin and binding is essentially irreversible.

2.12.2. Materials

The following general materials and reagents have been used in all the IHC experiments: standard microtome, xylene, absolute alcohol, alcohol 90%, alcohol 70%, distilled water, Giles haematoxylin, 1% acid alcohol, 0.3% H₂O₂ in methanol or water, Phosphate-buffered saline (PBS) buffer ,

eosin Y solution, xylene based mounting medium, staining jar , slide rack, glass slides and cover slips.

In addition, other special materials have been used for the IHC experiments: immunohistochemistry staining chamber for incubating the section, normal horse serum, primary antibody which was unique for every experiment, advanced avidin/biotin secondary antibody (Universal VECTASTAIN Elite ABC Kit, VECTOR LABORATORIES, USA) and DAB detection kit (ImmPACT DAB Peroxidase Substrate, VECTOR LABORATORIES, USA).

Moreover, the following materials and reagents have been used for the Heat-induced epitope retrieval (HIER) process: Domestic (850W) or scientific microwave, microwaveable vessel with slide rack to hold approximately 400-500 ml, and HIER buffer (10mM Sodium Citrate, 0.05% Tween 20, pH 6.0). The latter HIER buffer has been prepared as follows: 2.94 g of Tri-sodium citrate dihydrate were mixed to be dissolved in 1000 ml of distilled water; afterwards 0.5 ml was added of Tween 20 and mixed well. The pH was adjusted to 6.0 with 1N HCl.

2.12.3. Method

Firstly, the tissue block were cut by a slandered microtome to the desired thickness for IHC which is ideally 5 microns , and affixed onto the glass slide, then the sections were put in a 40°C water bath and picked up using glass slides and left to dry overnight.

Before proceeding with the staining protocol, the slides have been deparaffinized and rehydrated as incomplete removal of paraffin can cause poor staining of the section. To accomplish the step of deparaffinization the slides were placed in a rack, and the following washes performed with 2 washes in Xylene for 5 minutes each, and then 2 washes in absolute ethanol for 5 minutes; 5 minutes in 95% ethanol;

and then 5 minutes 70% ethanol. Afterwards the slides were put in running cold tap water for 10 minutes.

The slides were kept then in the tap water until ready to perform antigen retrieval. At no time from this point onwards the slides left to dry as drying out can induce non-specific antibody binding and therefore high background staining which will make the assessment of staining difficult or impossible sometimes.

Unless a different antigen retrieval method is stated on the primary antibody datasheet, the optimal HIER method for each antigen has been optimised experimentally.

At this stage, the antigen retrieval buffer was added to the microwaveable vessel in a volume of antigen retrieval solution sufficient to cover the slides by at least a few centimetres as we were using a non-sealed vessel to allow for evaporation during the boiling process. Then the slides were removed from the tap water and placed in the microwaveable vessel. The vessel was placed then inside the microwave. Because we were using a domestic microwave, full power had been used until the solution came to the boil. The solution was boiled for 20 minutes from this point.

When 20 minutes has elapsed, the vessel was removed and the cold tap water has been run into it for 10 minutes. This allowed the slides to cool enough so they may be handled, and allowed the antigenic site to re-form after being exposed to high temperature. Afterwards, quenching of endogenous peroxidase activity has been applied by incubating the sections for 30 minutes in 0.3% H_2O_2 in either methanol or water. Then the slides have been washed in PBS buffer for 5 minutes. To avoid non-specific staining the sections have been incubated for 20 minutes with diluted normal blocking serum, which was prepared from the species in which the secondary antibody is made, which was 10% horse serum in all the experiments, which took place in this study. Then the excess of

normal diluted horse serum has been blotted. At this stage, the sections have been incubated for 30 minutes with primary antibody diluted in PBS buffer, before being washed in PBS buffer for 5 minutes. Thereafter, the slides have been incubated for 30 minutes with diluted biotinylated secondary antibody solution, and washed afterwards for 5 minutes in PBS buffer. Then sections have been incubated for 30 minutes with VECTASTAIN® ABC Reagent, and washed afterwards in PBS buffer for 5 minutes. To achieve the desired stain intensity the sections can be incubated in peroxidase substrate solution using the DAB detection kit. As soon as the desired stain intensity is achieved while looking at the slides under the light microscope, the reaction should be stopped and the sections moved to jar where they can be rinsed very gently under tap water. Then the sections need to be counterstained in Giles haematoxylin for 5 minutes then rinsed under tap water for 5 minutes. Afterwards, the staining differentiation was achieved by immersing the sections in 1% acid alcohol 3 times. Then the sections can be moved at this stage for final dehydration step by immersing the slides in 70% alcohol for 3 minutes, followed by 90% alcohol for 3 minutes and finally 2 changes of absolute alcohol for 3 minutes each. The slides were cleared in 2 changes of xylene for 5 minutes each. Afterwards the slides were mounted with xylene based mounting medium and allowed to dry overnight.

2.13. COLORIMETRIC *in situ* APOPTOSIS ASSAY

2.13.1. Introduction

Apoptosis is characterized by specific morphological features, including membrane blebbing, nuclear and cytoplasmic shrinkage, and chromatin

condensation. Cells undergoing apoptosis fragment into membrane bound apoptotic bodies that are readily phagocytosed and digested by macrophages or neighbouring cells without generating an inflammatory response. This is in contrast to the type of cell death known as necrosis, which is characterized by cell swelling, chromatin flocculation, loss of membrane integrity, cell lysis and generation of a local inflammatory reaction.

The *in situ* apoptosis Terminal deoxynucleotidyl transferase dUTP Nick End Labelling (TUNEL) system end-labels the fragmented DNA of apoptotic cells. Biotinylated nucleotide is incorporated at the 3'-OH DNA ends using the Terminal Deoxynucleotidyl Transferase, Recombinant, (rTdT) enzyme. Horseradish peroxidase-labeled streptavidin (Streptavidin HRP) is then bound to these biotinylated nucleotides, which are detected using the peroxidase substrate, hydrogen peroxide, and the stable chromogen, diaminobenzidine (DAB). Using *in situ* apoptosis TUNEL system, apoptotic nuclei are stained dark brown.

2.13.2. Materials

DeadEnd™ Colorimetric TUNEL System (Promega Cat.# G7130) has been used for apoptosis detection in addition to the following materials and reagents: phosphate buffered saline (PBS), 0.3% hydrogen peroxide for blocking endogenous peroxidases, 4% paraformaldehyde, and mounting medium.

We also used some additional materials for processing the paraffin embedded sections during the TUNEL experiments: xylene, ethanol (100%, 95%, 85%, 70% and 50%) diluted in deionized water, 0.85% NaCl solution, proteinase K buffer, DNase I (RQ1 RNase-Free DNase, Promega Cat.# M6101), and DNase buffer.

2.13.3. Method

The paraffin-embedded sections have been deparaffinized by immersing the slides in fresh xylene in a Coplin jar for 5 minutes at room temperature. The latter step has been repeated for 5 minutes again. The slides have been washed by immersing the slides in 100% ethanol for 5 minutes at room temperature in a Coplin jar. The latter step has been repeated for 3 minutes. Then, the samples have been rehydrated by sequentially immersing the slides through graded ethanol washes (95%, 85%, 70% and 50%) for 3 minutes each at room temperature. Then the slides have been washed by immersing the slides in 0.85% NaCl for 5 minutes at room temperature. Afterwards the slides have been immersed in PBS for 5 minutes at room temperature. To fix the tissue sections they have been immersed in 4% paraformaldehyde solution or 10% buffered formalin in PBS for 15 minutes at room temperature. The slides have been washed twice in in PBS for 5 minutes at room temperature. At this stage, a 20 µg/ml Proteinase K solution has been prepared from the 10 mg/ml Proteinase K stock solution by diluting 1:500 in PBS. 100 µl of the 20 µg/ml Proteinase K solution have been added to each slide to cover the tissue section. The slides have been incubated at this stage for 10–30 minutes at room temperature. After the incubation time is complete, the slides have been washed by immersing them in in PBS for 5 minutes at room temperature in a Coplin jar. The tissues have been refixed at this stage by immersing the slides in 4% paraformaldehyde solution for 5 minutes at room temperature.

The slides have been washed at this stage twice by immersing the slides in PBS for 5 minutes at room temperature. The excess of liquid have been removed by tapping the slides. Then the tissues have been covered with 100 µl of Equilibration Buffer for 5–10 minutes. While the sections are equilibrating, thaw the Biotinylated Nucleotide was thawed and mixed on

ice to prepare sufficient rTdT reaction mix for all experimental and control reactions. One hundred microliters of reaction mix per slide were adequately able to cover the section. Then tissue papers were used to blot around the equilibrated areas then 100 μ l of rTdT reaction mix were added to the sections on a slide. The sections were covered then with coverslips to ensure even distribution of the reagent. Then the slides were incubated at 37°C for 60 minutes inside a humidified chamber to allow the end-labeling reaction to occur.

At this stage the 20X SSC was diluted to 1:10 with deionized water. Remove the Plastic coverslips have been removed afterwards and the reactions were terminated by immersing the slides in 2X SSC in a Coplin jar for 15 minutes at room temperature. At this stage, the slides were washed by immersing the slides twice in fresh PBS for 5 minutes at room temperature to remove unincorporated biotinylated nucleotides. The endogenous peroxidases have been blocked at this stage by immersing the slides in 0.3% hydrogen peroxide in PBS for 3–5 minutes at room temperature. The slides have been washed twice afterwards in PBS for 5 minutes at room temperature. The Streptavidin HRP solution has been diluted 1:500 in PBS, and 100 μ l of which have been added to each slide and incubate for 30 minutes at room temperature. Then the slides have been washed twice by immersing the slides in PBS for 5 minutes at room temperature. The DAB solution components have been combined just prior to use. For the latter step, 50 μ l of the DAB Substrate 20X Buffer have been added to 950 μ l of deionized water. Then both have been add 50 μ l of the DAB 20X Chromogen and 50 μ l of Hydrogen Peroxide 20X then the DAB solution was ready. At this stage, 100 μ l of DAB solution have been added to each slide and left to develop until there is a light brown background. The slides have been rinsed then several times in deionized

water. The slides were then ready for mounting in permanent mounting medium.

To prepare negative controls a control incubation buffer was prepared without rTdT Enzyme by combining 98 μ l of Equilibration Buffer, 1 μ l of Biotinylated Nucleotide Mix and 1 μ l of autoclaved, deionized water. On the other hand, to prepare positive controls we prepare a positive control slide by treating a sample with DNase I to cause DNA fragmentation. DNase I treatment of the fixed cells results in fragmentation of the chromosomal DNA and exposure of multiple 3'-OH DNA ends at which Biotinylated Nucleotides can be incorporated. This generally results in the majority of the treated cells demonstrating peroxidase labeling.

2.14. STATISTICAL ANALYSIS

Data was collected in a Microsoft Excel file. Statistical analysis was performed using SPSSTM computer software (IBM Inc.). Non-parametric tests such as Mann-Whitney U and Kruskal Wallis tests were the main tests used for general comparison of data. Simple linear regression and Chi² test as well as fisher exact test were also used where necessary. Survival/outcome was analysed using Kaplan Meier plots and estimates of survival curves were compared with Mantel-Cox test.

CHAPTER 3: MOLECULAR STUDIES RESULTS

3.1. TESTING THE STUDY AND CONTROL GROUPS BY PCR

The experiments on the total PCa and BPH populations using the MY09/11 degenerate primers have revealed that 43 cases were positive out of 123 cases in the PCa study group, while 25 cases out of 267 BPH control cases were positive. Also, the experiments on the total PCa and BPH populations using the GP5+/GP6+ consensus primers resulted in identical results to the results of MY09/11 degenerate primers. The experiments using nested MY/GP nested PCR method did not show any different results from the MY09/11 (Figure 3-1) or GP5+/GP6+ (Figure 3-2) PCR assays. On the other hand, all the positive cases in the PCa and BPH groups came up positive with the SPF1/2 multiplex PCR assay (Figure 3-3), in addition to 6 PCa and 3 BPH cases from the ones which came up negative with the GP5+/GP6+ and MY09/11 primer sets as well as the MY/GP nested PCR (Table 3-1). The statistical analysis of the results of each set of primers by non-parametric test for independent samples using Mann-Whitney U test for two independent groups showed that the results are statically significant with the p-value = 0.0011 for each of the MY09/11, GP5+/GP6+, MY/GP results on one hand and the SPF1/2 results on the other hand.

Table 3-1: HPV positive results by conventional, nested, and multiplex PCR.

| Primers | Group | I- PCa | I- BPH | II- PCa | II- BPH | III- PCa | III- BPH | Total PCa | Total BPH |
|---------------------------------|-------|-----------|-----------|------------|------------|-------------|-------------|-----------|-----------|
| GP5+/GP6+ (conventional PCR) | | 4 | 5 | 21 | 8 | 12 | 9 | 37(30%) | 22 (8.2%) |
| MY09/11 (conventional PCR) | | 4 | 5 | 21 | 8 | 12 | 9 | 37 (30%) | 22 (8.2%) |
| SPF1/2 (multiplex PCR) | | 4 | 6 | 24 | 8 | 15 | 11 | 43(34.9%) | 25 (9.3%) |
| MY/GP (nested PCR) | | 4 | 5 | 21 | 8 | 12 | 9 | 37 (30%) | 22 (8.2%) |
| Total Number of Cases | | 49 | 99 | 43 | 77 | 31 | 91 | 123 | 267 |

I = Middle Eastern population.

II = Caucasians population.

III = Afro-Caribbean population.

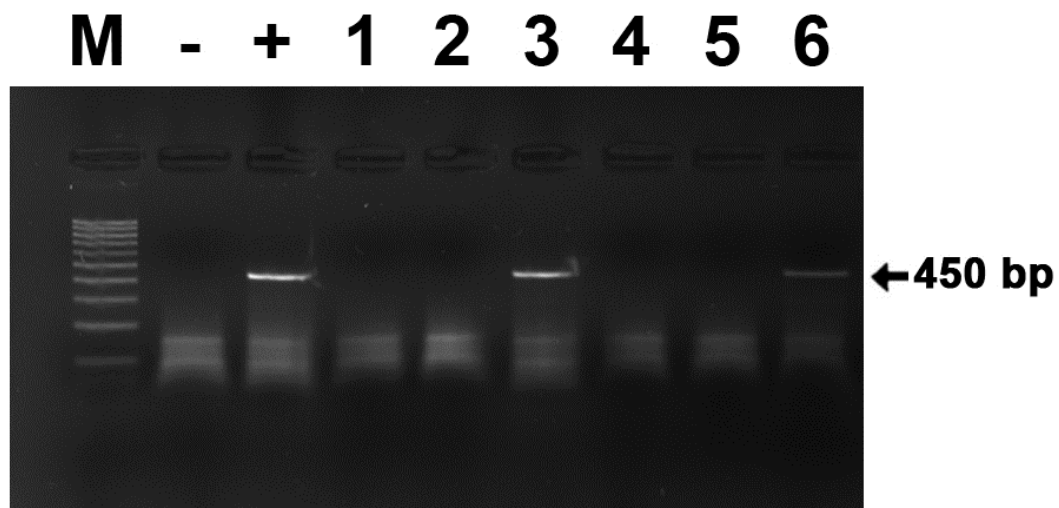


Figure 3-1: MY09/11 PCR assay (450bp). M = DNA molecular weight marker. Sample of positive PCR- reactions are seen in specimens 3 and 6. + = positive control, - = negative control.

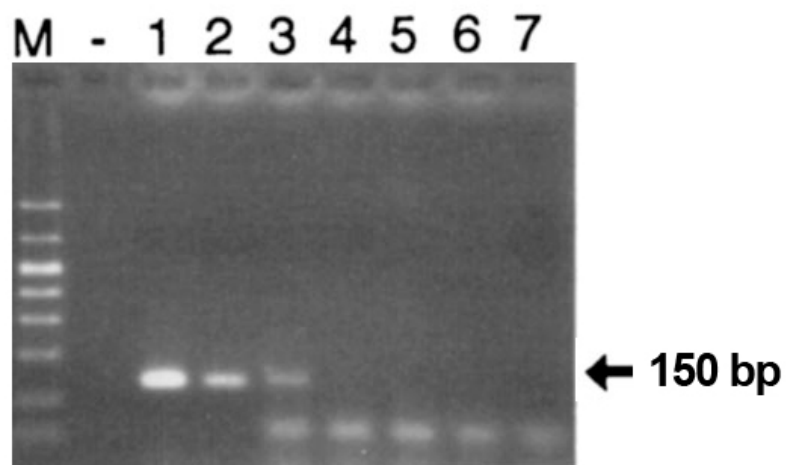


Figure 3-2: GP5+/GP6+ PCR assay (150bp). M = DNA molecular weight marker. Sample of positive PCR- reactions are seen in specimens 2 and 3. 1 = positive control, - = negative control.

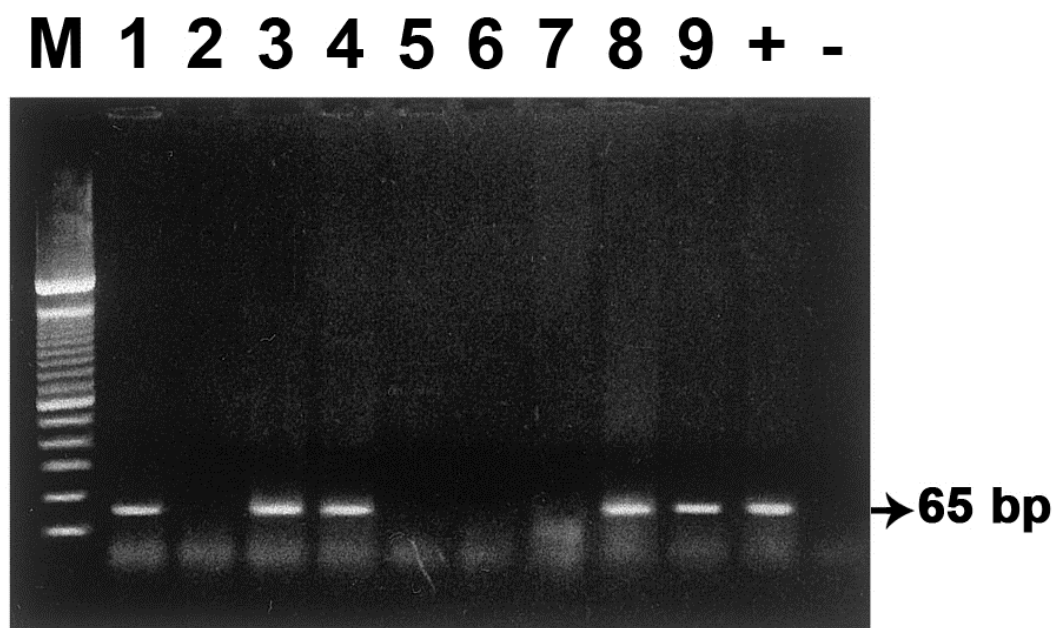


Figure 3-3: SPF1/2 PCR assay (65bp). M = DNA molecular weight marker. Sample of positive PCR- reactions are seen in specimens 1, 3, 4, 8 and 9. 1 = positive control, - = negative control

3.2. HPV GENOTYPING BY REVERSE HYBRIDISATION LINE

PROBE ASSAY

We used the reverse hybridization line probe assay method to genotype the HPV- positive samples. In this technology part of the L1 region of the HPVs genome is amplified using biotinylated SPF1/2 primer set in a multiplex PCR method, then the denatured biotinylated amplicons are hybridised with specific oligonucleotide probe immobilised on a test strip [272]. High-risk HPVs were detected in 30 of 43 (69.9%) of the total PCa cases, which came up positive by the SPF1/2 multiplex PCR assay for HPV DNA. Also, 16 of 25 (64%) BPH samples, which were positive for HPV-DNA, showed high-risk HPV infection. Low-risk HPVs were detected in 13 of 43 (30.2%) of the PCa cases, and 9 of 25 (36%) of the BPH specimens which were positive for HPV-DNA. It was notable that HPV-18 was the predominant HPV type in PCa cases while HPV 16 was predominant in BPH samples (Table 3-2). These different frequencies of the high-risk and low-risk HPV infection within the total study and control groups are of statistical significance (Fischer's exact test with Woolf's approximation, $p=0.0013$).

Table 3-2: HPV types in HPV-DNA-positive prostatic carcinoma and benign prostatic hyperplasia samples.

| HPV Types | | | | | |
|------------|---------------|--------|----------------|--------|--------------------|
| | Low Risk HPVs | | High Risk HPVs | | Total HPV positive |
| | HPV-6 | HPV-11 | HPV-16 | HPV-18 | |
| PCa | 5 | 8 | 12 | 18 | 43 |
| BPH | 4 | 5 | 9 | 7 | 25 |

Furthermore, high-risk HPVs were detected in 30 of 123 (24.3%) of PCa cases in the total study group and 16 of 267 (5.9%) of BPH samples in the total control group. On the other hand, low-risk HPVs were detected in 13 of 123 (10.5%) and 9 of 267 (3.3%) of the total PCa and BPH samples respectively (Table 3-3). These results are statistically significant (chi-squared test, $p=0.011$).

Table 3-3: High-risk and low-risk HPV infection frequencies in the total prostatic carcinoma and benign prostatic hyperplasia samples.

| Group | Low-risk HPV | High-risk HPV | Total cases |
|-------|--------------|---------------|-------------|
| PCa | 13 (10.5%) | 30 (24.3%) | 123 |
| BPH | 9 (3.3%) | 16 (5.9%) | 267 |

Moreover, high-risk HPVs were found in 4%, 44% and 29% in the PCa subgroups of the Middle Eastern, Caucasian and Afro-Caribbean patients respectively. On the other hand, high-risk HPVs were found in 2%, 9% and 7.6% in the BPH subgroups of the Middle Eastern, Caucasian and Afro-Caribbean patients respectively (Table 3-4). The analysis of these results by chi-squared test showed that they are statistically significant ($p=0.0018$).

Table 3-4: High-risk and low-risk HPV infection in the study and control sub-groups.

| Group | I-PCa | I-BPH | II-PCa | II-BPH | III- PCa | III-BPH |
|-----------------------|--------|--------|-----------|----------|-----------|----------|
| High Risk HPVs | 2 (4%) | 2 (2%) | 19 (44%) | 7 (9%) | 9 (29%) | 7 (7.6%) |
| Low Risk HPVs | 2 (4%) | 4 (4%) | 5 (11.6%) | 1 (1.2%) | 6 (19.3%) | 4 (4.3%) |
| Total number of cases | 49 | 99 | 43 | 77 | 31 | 91 |

I = Middle Eastern population.

II = Caucasians population.

III = Afro-Caribbean population.

The frequencies of high-risk HPV within the PCa study sub-groups were 30%, 46.6% and 23.4% for Gleason 6, Gleason 7 and Gleason 8-10 respectively. (Table 3-5). These results indicate that there is no association between Gleason scoring and HPV infection within the study group samples; (Fischer's exact test with Woolf's approximation, $p = 0.09$).

Table 3-5: The association between the HPV-positive cases and Gleason scoring.

| Group | *I PCa | **II PCa | ***III PCa | Total PCa |
|--------------|--------|----------|------------|------------|
| Gleason 6 | 1 | 5 | 3 | 9 (30%) |
| Gleason 7 | 2 | 7 | 5 | 14 (46.6%) |
| Gleason 8-10 | 0 | 4 | 3 | 7 (23.4%) |

*I-PCa= Prostate cancer subgroup of the Middle Eastern population.

**II-PCa= Prostate cancer subgroup of the Caucasian population.

***III-PCa= Prostate cancer subgroup of the Afro- Caribbean population.

We found that the frequencies of high-risk HPVs were 16.9%, 45%, 28.1% in the T1-2N0M0, T3-4N0/xM0 and T2-4Nx/+M1 stages in the total study group respectively (Table 3-6). The statistical analysis of these results by Fischer's exact test with Woolf's approximation indicates that there is no association between the high risk HPV DNA existence and the staging of the prostate cancer in the total study group ($p=0.8$).

Table 3-6: association between tumour stage and infection with high-risk HPV.

| TNM Staging | Number of high- risk HPV infection cases | Total number of cases |
|------------------------------------------------|------------------------------------------|-----------------------|
| Organ-confined disease T1-2N0M0 | 12 (16.9%) | 71 |
| Locally advanced disease T3-4N0/xM0 | 9 (45%) | 20 |
| Metastatic T2-4Nx/+M1 | 9 (28.1%) | 32 |

Furthermore, it has been found, using chi-square test that there was no statistically significant difference ($p=0.064$) in the PSA level among the groups of PCa which are HPV-negative and the ones which are positive for low-risk or high-risk HPV infections (Table 3-7).

Table 3-7: The mean of PSA value in HPV-negative, low-risk HPV, and high-risk HPV infections prostate cancer cases.

| Groups | PSA(μ g/L) mean in HPV-negative PCa | PSA(μ g/L) mean in low-risk HPV cases | PSA(μ g/L) mean in high-risk HPV cases |
|----------------|------------------------------------------|--------------------------------------------|---------------------------------------------|
| Group 1 | 6.1 | 5.9 | 5.4 |
| Group 2 | 8.9 | 7.8 | 7.6 |
| Group 3 | 8.2 | 8.8 | 7.9 |

Group I = Middle Eastern population.

Group II = Caucasians population.

Group III = Afro-Caribbean population.

Likewise, Using Kaplan-Meier survival estimates, there was no significant difference in survival between the PC subgroups, which were negative for HPV infection, the group, which was positive for low-risk HPV infections, and finally the group, which was positive for the high-risk HPV infections. The survival estimates have been summarized in a Kaplan-Meier survival plot (Figure 3-1).

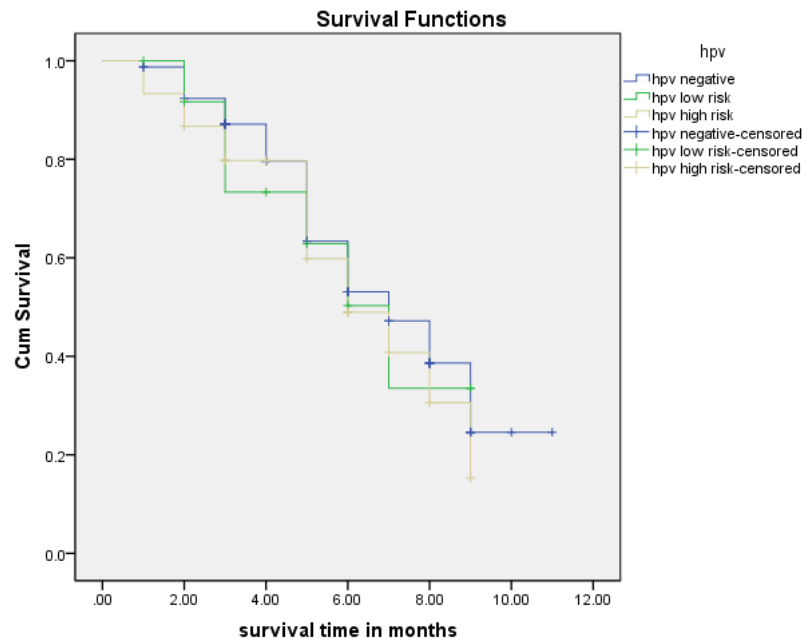


Figure 3-4: Kaplan-Meier survival plot for the three PCa subgroups: a) negative for HPV DNA; b) positive for low-risk HPV DNA; d) positive for high-risk HPV DNA.

3.3. QUANTITATIVE STUDIES RESULTS

High-risk HPV viral DNA load was measured in each SPF1/2 multiplex PCR assay product, which showed positivity for high-risk HPV DNA. Each sample was tested anonymously using the technology of the Microchip Electrophoresis System for DNA/RNA Analysis (MCE®-202 MultiNA). The results have shown that the average high-risk viral DNA concentration in the PCa samples was 1.40 ng/ μ l, while the average high-risk viral DNA concentration was 0.97 ng/ μ l in the BPH samples (Table 3-8; Figure 3-5).

Table 3-8: The average high-risk HPV viral DNA concentration in the study and control groups.

| Group | Total PCa | Total BPH |
|----------------------------------------|-----------|-----------|
| Number of high-risk HPV-positive cases | 30 | 16 |
| High-risk HPV DNA ng/μl (average) | 1.40 | 0.97 |

I = Middle Eastern population.

II = Caucasians population.

III = Afro-Caribbean population.

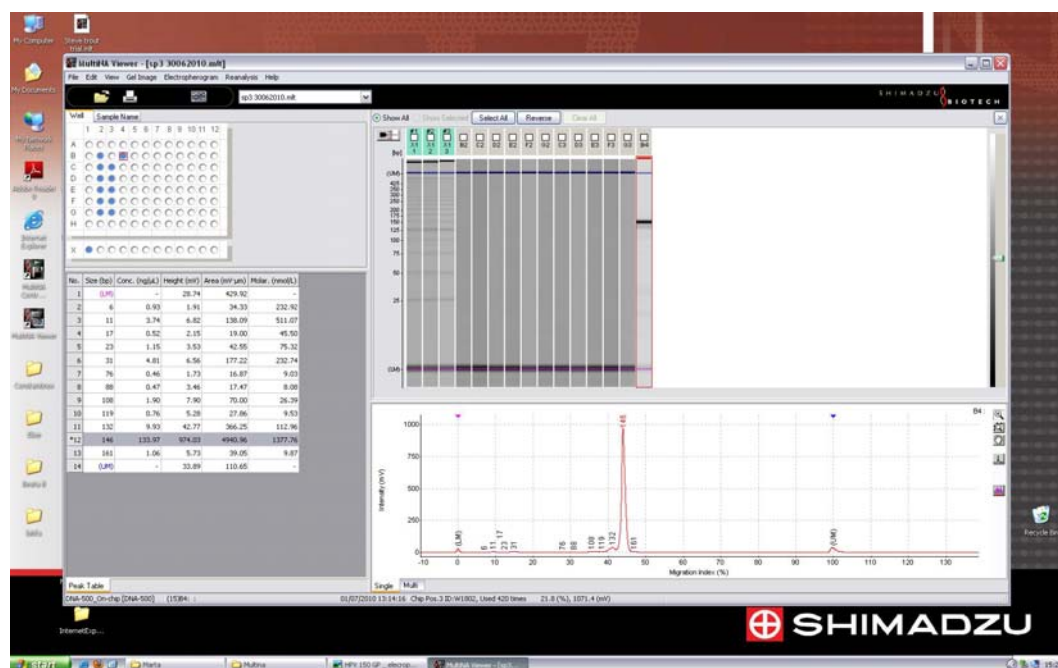


Figure 3-5: Print Screen from Microchip Electrophoresis System for DNA/RNA Analysis (MCE®-202 MultiNA) interface showing the DNA concentration in sample no. 12 as 133.97 ng/μl.

Furthermore, it has been shown that the average viral DNA concentration in the high-risk HPV positive cases was 1.54 ng/ μ l, 1.27 ng/ μ l and 1.41 ng/ μ l in Gleason 6, Gleason 7 and Gleason 8-10 prostate cancer cancers respectively. These results are not statistically significant using Fischer's exact test with Woolf's approximation ($p=0.89$), which indicates that there is no association between the HPV DNA concentration and extent of the prostate cancer grade in the total study group (Table 3-9).

Table 3-9: The association between Gleason scoring and the high- risk HPV viral DNA concentration.

| Gleason Scoring | HPV DNA ng/ μ l (Average) | Total Number of cases |
|-----------------|----------------------------------|--------------------------|
| Gleason 6 | 1.54 | 9 |
| Gleason 7 | 1.27 | 14 |
| Gleason 8-10 | 1.41 | 7 |

Additionally, it has been found that the average viral DNA concentration in cases which showed positivity for the high- risk HPVs was 2.21 ng/ μ l, 1.89 ng/ μ l and 2.18 ng/ μ l in T1-2N0M0, T3-4N0/xM0 and T2-4Nx/+M1 prostate cancer cases respectively. These results are not statistically significant using Fischer's exact test with Woolf's approximation ($p=0.79$), which shows that there is no association between the HPV DNA concentration and the extent of prostate cancer metastasis in the total study group (Table 3-10).

Table 3-10: The association between TNM staging and the high-risk HPV viral DNA concentration.

| TNM Staging | HPV DNA ng/μl (average) |
|------------------------------------------------------|------------------------------------|
| Organ-confined disease T1-2N0M0 | 2.02 |
| Locally advanced disease T3-4N0/xM0 | 0.97 |
| Metastatic T2-4Nx/+M1 | 1.21 |

3.4. GENETIC POLYMORPHISM STUDIES

The *p53* gene codon 72 polymorphism detection technique was based on differences in fragment size for the two forms of the codon 72 of the *p53* gene, which are: the *p53*-Pro allele that yields a 177-bp fragment as well as the *p53*-Arg allele which yields a 141-bp fragment by allele-specific PCR [264].

The Arg/Arg genotype of the *p53* codon 72 polymorphism was found in 62 (50.4%) of the total 123 PCa cases, while the the Arg/Pro genotype was found in 57 (46.4%) and the Pro/Pro genotype was found in 4 (3.2%). On the other hand, out of the total 267 BPH cases 126 (47.1%) were Arg homozygous, 12 (4.4%) Pro homozygotes, and 129 (48.5%) remaining cases were heterozygous (Table 3-11; Figure 3-6).

Table 3-11: Genotypes and frequencies of codon 72 *p53* polymorphism variants in PCa and BPH cases.

| <i>p53</i> codon 72 allele | PCa | BPH |
|----------------------------|------------|-------------|
| Arg/Arg | 62 (50.4%) | 126 (47.1%) |
| Pro/Pro | 4 (3.2%) | 12 (4.4%) |
| Pro/Arg | 57 (46.4%) | 129 (48.5%) |
| Total | 123 | 267 |

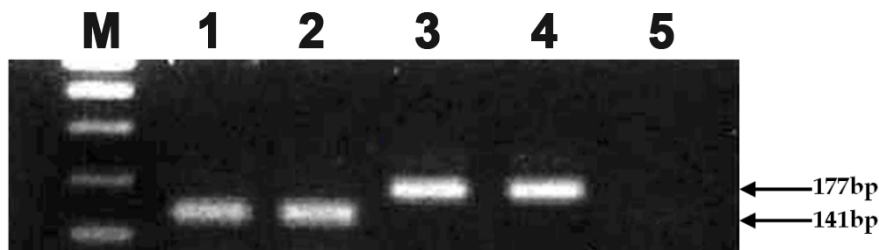


Figure 3-6: *p53* gene codon 72 polymorphism PCR assay. *p53*-Pro allele yields a 177-bp fragment which can be seen in samples 3 and 4. *p53*-Arg allele yields a 141-bp fragment which can be seen in samples 1 and 2. M = DNA molecular weight marker. 5 = negative control.

Furthermore, the *p53* codon 72 allelic frequencies amongst patients with HPV-positive and HPV-negative carcinomas were compared in order to evaluate whether an association between *p53* Arg homozygosity and high-risk HPV-positivity might exist. Out of 30 patients with high-risk HPV-positive carcinomas, 14 were Arg homozygotes, 3 were Pro homozygotes and 13 were Arg/Pro heterozygotes. On the other hand from the 80 patients with HPV-negative carcinomas, 44 were Arg homozygous, 4 were Pro homozygotes and 32 were heterozygotes (Table 3-12).

The statistical analysis using Fischer's exact test with Woolf's approximation test the samples showed that there was no significant difference ($p=1.00$) in the frequency of *p53* Arg homozygosity between HPV-positive and HPV-negative prostate carcinomas.

Table 3-12: Frequencies of codon 72 *p53* polymorphism variants in high-risk HPV-positive and HPV-negative prostate carcinomas

| <i>p53</i> codon 72 allele | Prostate Carcinomas | |
|----------------------------|---------------------|----------|
| | high-risk HPV (+) | HPV (-) |
| Arg/Arg | 14 (46.6%) | 44 (55%) |
| Pro/Pro | 3 (10%) | 4 (5%) |
| Pro/Arg | 13 (43.4%) | 32 (40%) |
| Total | 30 | 80 |

3.5. DISCUSSION

Numerous broad-spectrum PCR primer sets have been designed for detection of multiple HPV types by a single PCR assay [273]. However, none of the currently available universal primer sets is able sufficiently to detect all HPV genotypes, as recent studies have shown that the overall prevalence of HPV can be underestimated considerably if only a single PCR assay is used [274].

To overcome the problem of underestimating the prevalence of HPV infection in this research three primer sets have been used to detect the HPV infections in the extracted genomic DNA (Table); GP5+/GP6+ consensus primers [254], and MY09/11 degenerate primers [255] , in addition to broad-spectrum multiplex PCR primer set SPF1/2 [256]. These

three PCR assays were designed to amplify distinct highly conserved L1 ORF regions of the HPV genome with different amplicon sizes (Figure). More than one primer set had been used because it has been reported that using a single primer set led to underestimating the prevalence of HPV infection in many studies, and simultaneous use of more than one primer set is an important tool for the detection of HPV DNA [275]. Also, because it has been reported that the efficacy of each primers set for detection of human papillomavirus DNA by PCR in paraffin-embedded seemed to be inversely correlated to the size of the amplicon produced [273].

Furthermore, to increase the sensitivity of HPV detection using the consensus and degenerate primers mentioned above, the samples were screened for the presence of HPV using the standard nested PCR approach, consisting of the MY09/11 degenerate primer set as outer primers and the GP5+/GP6+ consensus primer set as inner primers. This nested PCR method has been collectively called MY/GP [257].

The oncogenic role of HPV in cancers in several anatomical places, such as prostate, bladder, oesophagus, larynx and oral cavity, has been proposed, based on the detection of HPV-DNA in tissue samples taken from these cancers [85, 87, 212, 276], but these reports have been somewhat controversial so far [212, 276]. Since 1990, almost 32 publications have been conducted to detect HPV in PCa cases [212]. These studies have been carried out in different countries with different detection methodologies and have mostly been case – control studies [277-281]. Some studies have concluded that prostate carcinomas are related to HPV infection and have shown that the presence of HPV in PCa was varied between 2% to 80%. The most common types of HPV detected in PCa cases were HPV types 16, 18, 33 and 31 [217, 277, 278, 282-285]. On the other hand, some

researchers have reported that HPV is equally prevalent in PCa and BPH and even in normal prostate tissue [283, 286, 287] and some studies have been unable to detect HPVs in normal and cancer prostate tissues [288, 289].

In the current study, we detected that there is significant difference in HPV positivity between prostate carcinomas and hyperplasias in the analysed samples. The detected oncogenic types of HPV, in particular HPV types 16 and 18, are confirmed to be the major causative agent of both squamous and adenocarcinoma of the cervix [290-293], as well as intraepithelial neoplasia of the anogenital tract, which are precursor lesions of anogenital cancers [294-296].

Our study showed that there was significant difference between PCa and BPH specimens regarding the presence of HPV-DNA. We found high-risk HPVs in 24.3% of PCa and 10.5% of BPH samples. Highly variable frequencies ranging between 2% and 53% have been reported for the detectability of high-risk HPVs in tissue obtained from BPH and prostate cancer specimens [297]. These differences could be attributed to different methodologies of tissue handling and the sensitivity of detection methods. Also, another possible explanation for the divergent frequencies of high-risk HPV positivity in prostate cancer samples could be attributed to sociological, geographical, environmental, and genetic heterogeneities causes, which have not been researched yet. In addition, low educational level population revealed a higher prevalence of HPV infection in several studies, but this has not been investigated so far in the prostate cancer cases, which may deserves further multidisciplinary academic work in the future [298]. These divergent frequencies were noted in the current study

as high-risk HPV detection rate was 2%, 44%, and 29% of the PCa cases of the Middle Eastern, Caucasian, and Afro-Caribbean subgroups respectively. This might be because of the genetic makeup of the UAE nationals which comprise the cases in the Middle Eastern Group is mostly Asian with low penetration of prostate cancer genes, dietary intake devoid of saturated fats and consumption of a protective Mediterranean diet, in addition to the fact that all males in UAE are circumcised, usually in their early neonatal life. Furthermore, being raised in a conservative social system that strictly prohibits extramarital sexual activities has led to low prevalence of STDs, and consequently low HPV infection rate.

Moreover, our results are consistent with the data reported by Leiros et al., who found that 41.5% of the DNA extracted from paraffin embedded 41 PCa cases were HPV-positive [213]. However, our results disagree with an independent study by Bergh et al., which was conducted in Sweden and did not find HPV sequences in any of the studied 352 prostate tissue samples [289].

Discrepancies could be also explained by technical differences in the sensitivity of the tests used for detection the HPV DNA or can be attributed to the differences in sexual behaviour in the study populations, as HPV prevalences in healthy men demonstrate large variations worldwide, ranging between 4% and 64% [218].

We found that the oncogenic high risk HPVs are predominantly type 16 and 18, which may suggest that the HPV association with PCa cases is due to specific type of HPV, which is consistent with previous reports [299, 300]. However, the presence of low-risk HPV 11 and HPV 6 DNAs in the

PCa samples indicates the possibility that HPV can infect the prostate, but these infections may also have no effect on the carcinogenic process.

Most of the samples studied in this research were TURP specimens, which may be the underlying reason of a possible viral contamination of prostate tissue by HPV from neighbouring tissues – namely the urethra- during the sampling procedure. However, the significant differences in virus prevalence amongst the case and control groups minimize the contamination possibility. Based on this hypothesis it might be recommended that radical prostatectomy is the best tissue source for researching HPV prevalence in PCa cases, as well as careful microdissection of the neoplastic sample from the surrounding benign tissues. In this study radical prostatectomy samples could not be obtained. Also, microdissection was not possible because of limited tissues remaining in the paraffin blocks. Likewise, HPV contamination from the anal epithelium might be a common event during biopsy taking and sample manipulation because of the inevitable contact with the anal epithelium during the process of taking the biopsy. This assumption can be supported in this study by the fact that both carcinomas and hyperplasias showed some degree of HPV detection for both high-risk and low-risk HPVs.

Moreover, small latent carcinomas, which occur in 30–50% of all prostates [300], might remain undetected within single BPH specimens during routinely performed histopathological examination [301]. Therefore, the high HPV level that was observed in our BPH samples may result from unrecognized microscopic carcinoma.

A number of studies have compared the ability of different primer sets to detect HPV DNA in clinical materials obtained from cervical and

anogenital cancers [273, 302-305]. From these studies a few basic rules can be established. (i) Each set of HPV-PCR primers needs to be evaluated in a wider clinical context using other histopathological and biochemical markers since theoretical predictions may not accurately reflect the clinical status of the virus in the tissue [275]. (ii) The overall prevalence of HPV DNA in any given collection of clinical materials is underestimated if only a single detection method is used [257, 274, 306]. (iii) For samples processed from tissues originally fixed in formaldehyde and embedded in paraffin, the smaller the PCR amplification product, the higher the rate of HPV DNA detection [273, 304].

In this study we have compared the two major primer sets used for PCR amplification of HPV DNA in a group of PCa and BPH samples obtained from men from three different ethnic groups. The MY09/MY11 primer set has been used predominantly in epidemiologic studies originating in North and South America and Asia, whereas the GP5+/GP6+ primer system has been used primarily in Europe [304]. Each PCR method showed a high level of reproducibility when compared to itself, as previously demonstrated with the MY09/MY11 assay in different laboratories [307, 308]. There was also good agreement in the detection of HPV DNA-positive and DNA-negative status between the latter two PCR methods [309]. In addition, it has been reported that each HPV primer set amplified some HPV types better than others, as the most marked differences were seen in the ability of MY09/MY11 degenerate primers to amplify HPV-35 and GP5+/GP6+ consensus primers to amplify HPV types 53 and 61 [304], but this has not been shown in our study as these subtypes have not been detected using either of the primers sets.

There were reports that women infected by HPV were detected more efficiently by MY09/11 than by GP5+/GP6+ according to the analytical sensitivity results calculated for each primer set, mainly in cases of multiple infections [304]. These findings were based on the fact that MY09/11 consisting of a system of degenerated primers thereby promoting the identification of multiple infections [310]. This hypothesis could not be tested in our study in PCa cases because we only established single infections subgroups with HPV subtypes, and subsequently showed identical concordance between the GP5+/GP6+ consensus primers and MY09/11 degenerate primers.

Several authors have recommended the use of frozen tissue instead of FFPE samples [7]. Other authors have argued that FFPE samples are a reliable DNA source as long as PCR products that are generated are smaller than 150 bp because of fixative-induced cross-links [1]. Nonetheless, it has been reported that HPV detection using PCR depends on the primer set that is utilized and unsuccessful amplification results are due to DNA damage caused during the process of formalin fixation and paraffin embedding [311]. Moreover, PCR assays yielded different results when different annealing temperatures were used. The most commonly used primer, MY09/MY11, which amplifies a highly conserved region of the L1 open reading frame, produced a 450-bp product in all of the human DNA samples at 40°C; however, increasing the annealing temperature to 55°C eliminated this effect [312]. To address the problem of annealing temperatures, we reviewed the studies that employed MY09/MY11 primers for the annealing temperature values. A vast majority of the studies used appropriate annealing temperature of 55°C except for one study that used 45°C [299]. Among the four studies that produced significant results, two used the MY09/MY11 primer. One of these studies

amplified the 450-bp fragment at 55°C [213], whereas the other study did not report the temperature that was used [313]. In the current research 54°C has been used as annealing temperature when MY09/MY11 have been used.

In this study we have combined GP5 +/GP6+ consensus primers and MY09/MY11 degenerate primers in a two-step amplification nested PCR to analyse further those cases that proved negative with either GP5 +/GP6+ or MY09 /MY11. We found that the MY/GP Nested PCR experiments gave identical results to GP5+/GP6+ consensus primers and MY09/11 degenerate primers systems. This result agrees with few previous reports which followed the same methodology of MY/GP Nested PCR for detecting HPV infections in cervical cancer specimens [314]. However, our results contradict with another report, which found that the use of the MY/GP nested PCR increased significantly the positivity rate of HPV DNA detection [257]. This divergence in the latter report might be due to improper optimisation of one of the two primers sets, which will result in higher sensitivity with the nested PCR in comparison with each individual primer due to the compensating annealing process with the other primer set in the second round of the nested PCR.

We found that the amplicon size of the SPF1/2 multiplex PCR system was only 65 bp, while the amplicon sizes of GP5 +/GP6+ and MY09/11 systems were 150 and 450 bp, respectively. It has been reported that the sensitivity of DNA detection by PCR is inversely related to the size of the amplicon, as the kinetics of the PCR reaction favour smaller amplicons [315]. Furthermore, as the efficacy of PCR also depends on the quality of the DNA extracted from the clinical specimen, some researchers reported

that detection with small amplicons will be advantageous, especially when using formalin-fixed, paraffin-embedded materials [273, 316]. Taken together, the small amplicon size may explain the high sensitivity of the SPF1/2 assay in our study. On the other hand, the SPF1/2 multiplex PCR system uses a mixture of defined primers for the SPF1 and SPF2 target regions. These regions are also used for GP5 +/GP6+ and MY09/11 priming, respectively. Because of the heterogeneity of the primer target sites, the SPF1/2 mixture of primers may be more effective than GP5 +/GP6+ and MY09/11 primers. Moreover, our results showed that the use of one consensus and degenerate HPV primer pair with paraffin-embedded tissues would underestimate the presence of HPV DNA in the studied population.

The prognostic significance of HPV DNA in genital neoplasms, in terms of tumour staging and grading as well as the patients survival rate, is controversial [317]. In a large series of cervical carcinoma, which comprised 291 cases, it was reported that HPV DNA status was not predictive of survival in the whole study population, although there was a significant association between HPV-18 and poor prognosis in early stage tumours [318]. However, another study found that patients with HPV-16 positive cervical tumours had significantly higher rates of pelvic metastasis to lymph nodes than patients with HPV-16 negative tumours [319]. Other studies also have shown an association between the presence of high-risk types and poor clinical outcome [320]. On the contrary, some investigators reported that the absence of HPV in the cervical tumour confers a worse prognosis [321]. One of these researchers analysed 106 patients with cervical carcinomas, and concluded that patients with no detectable HPV DNA sequences had a 2.6 times higher risk of overall recurrence and 4.5 times higher risk of distant metastases than did HPV

positive patients [322]. Another interesting report confirmed that HPV negative status and c-myc overexpression are two independent prognostic indicators related to the risk of distant metastases in cervical carcinoma [323]. In a trial to explain the latter reports it was suggested that in HPV negative tumours, *p53* and *pRb* may be mutated or deleted to account for functional inactivation of these important cell cycle regulators [321].

On the other hand, there were two reports addressed the relation between HPV and prognosis of penile carcinoma. Both reports compared the clinical outcomes of patients with HPV positive versus HPV negative tumours, and found that there is no difference in patient age, tumour staging, or the Kaplan–Meier estimates of survival [317, 324]. Those latter reports seem in line with the results we found in the current study which suggest that HPV status of the PCa primary tumour does not influence the tumour prognosis indicators. In sum, little is known regarding the mechanisms involved in the invasiveness and metastatic process of HPV positive carcinomas; and further studies are required to provide further insight into those mechanisms.

Although the data about the natural history of HPV infections are limited, the viral load in cervical cancer is presumably low during the latency period and first phase of infection but may increase over time, in parallel with the development of cytological aberrations [85, 87, 150, 186]. Several authors have reported a positive association between the viral burden of high-risk HPV with the high-grade intraepithelial lesion [23,24]. But, by contrast with the case-control researches in the cervical cancer cases [325], which examined the role of viral load in cervical cancer grade as well as stage, this study concluded that HPV viral load measured in the high-risk HPV positive PCa cases was not associated with the grade or stage of the PCa cases. There are several possible explanations for the conflicting

findings. First, comparison of HPV viral load values between studies is difficult because data are not given in terms of an absolute measure of viral genomes. Second, our study looked at an additive measurement of viral load for type 18 and 16 carcinogenic HPV types, whereas the previous studies focused only on type 16. Third, our results might differ from those of other studies in cervical cancer because of differences in sample anatomic origin and collection method which was mostly TURP and trans-rectal biopsies in our study, while the in the cervical cancer cases spatulas were probably used to collect samples which might led to different level of representation of cells infected with HPV from the transformation zone in cervical cancer.

Furthermore, It is well known that the first line of defence against the viral infection is the interferon response, as E6 HPV oncoproteins can inhibit the interferon pathway [326, 327], which can lead to more aggressive disease. However, the results from our research do not support this hypothesis as no association could be established between the grade of the tumour represented by Gleason scoring and the HPV status in the PCa samples.

Transrectal ultrasound-guided prostate biopsy is one of the most important methods used as a first confirmatory test in prostate cancer [328]. The main indication for prostate biopsy is elevation of the serum PSA level, together with unfavourable digital rectal examination result. However, the PSA level is not only elevated in men with prostate cancer [329]. Other diseases, including benign prostatic hyperplasia and symptomatic chronic prostatitis, as well as prostate manipulations, can increase the serum PSA level [330]. On the other hand, the prostate volume is variable and could result in variations in the serum PSA level in

men without prostate cancer [331]. Considering the wide range of situations that can increase the serum PSA level, many patients undergo prostate biopsy because of an elevated PSA level, and an important percentage of them do not have prostate cancer [332]. Also, according to published reports, prostate biopsies may fail to detect cancer in up to 30% of patients [333]; thus, many urologists recommend repeat biopsies to their patients, sometimes with more specimens taken in an attempt to find a possible previously undetected area of cancer [334]. All of these factors can contribute to an increase in the PSA level, and make it harder to predict which factor is the direct causative agent for the increase of the PSA level [335]. In addition, it has been reported that there was an association between prostate epithelium disruption and serum PSA increase [330] which might be, in theory, caused in the course of HPV infection within the prostatic tissues. But, also it has been concluded in the same study that the extension of the inflammatory infiltration incurred was not related to an increase in serum PSA [330]. However, in the current research there was no association between the PSA level and the status of HPV infections within the study group. That could be explained by the lengthy latency period of the HPV infections which does not induce any prominent disruption of prostatic epithelium, along with the fact that any real increase in the PSA level in an HPV-positive case might not be noticed in this research because of the other actions which may be taken during the course of assessing any prostatic pathology in the other cases which are HPV-negative. Therefore, the latter observation will make the statistical analysis difficult of the PSA levels as they cannot be analysed statistically for every individual case but rather they should be taken as a statistical value for a group of cases.

We assessed the allelic frequencies of the *p53* gene codon 72 polymorphism (Arg, Pro or Arg/Pro) in the cases of the study and control

groups in order to evaluate the possibility of increased cancer susceptibility associated with the Arg/Arg genotype. However, no statistically significant differences amongst the Arg/Arg and Arg/Pro genotypes frequencies could be established, neither by comparing patients with PCa and BPH nor between high-risk HPV-positive and High-risk HPV-negative carcinomas.

In cervical cancer, several studies of the *p53* codon 72 polymorphism have been accomplished after the initial report which concluded a higher cancer risk associated with the Arg allele [264]. Some of these researches disagreed with the original report [265] whereas others support it [336, 337]. In our assessment of the *p53* polymorphism at codon 72, we could not find an indication that the Arg allele confers a higher risk for prostate cancer, including those tissues positive for high-risk HPV. Moreover, it has been reported that the Pro/Pro genotype is associated with a reduced risk of developing prostate cancer [338]. However, in the current study we could not evaluate this hypothesis due to the low populational frequency of the rare Pro/Pro genotype in our study and control populations.

CHAPTER 4: MORPHOLOGICAL STUDIES RESULTS

4.1. TUMOUR GRADING USING GLEASON SCORING SYSTEM

All the study cancer subgroups have been examined to assess Gleason scoring in each case after being stained using H&E method and examined by light microscopy. Gleason scoring results are essential starting points to investigate any association between the grade of the prostate cancer cases and any of the other direct and indirect indicators of HPVs infections in the study and control groups.

The results have shown that the tumour grade within the Middle Eastern population were 34.6%, 30.8% and 34.6% for Gleason 6 (Figure 4-1), Gleason 7 (Figure 4-2) and Gleason 8-10 (Figures 4-3; 4-4) respectively. While it was 34.8%, 48.8% and 16.4% for Gleason 6, Gleason 7 and Gleason 8-10 respectively within the Caucasian population. As for the Afro-Caribbean population, the distribution was 29%, 54.8% and 16.2% for Gleason 6, Gleason 7 and Gleason 8-10 respectively (Table 4-1).

Table 4-1: Gleason scoring in the three cancer subgroups

| Group | I-PCa* | II-PCa** | III-PCa*** | Total Number of PCa Cases |
|----------------------------------|-------------|------------|------------|---------------------------|
| Gleason 6 | 17 (34.6%) | 15 (34.8%) | 9 (29%) | 41 (33.3%) |
| Gleason 7 | 15 (30.8 %) | 21 (48.8%) | 17(54.8%) | 53 (43%) |
| Gleason 8-10 | 17 (34.6%) | 7 (16.4%) | 5 (16.2%) | 29 (23.7%) |
| Total number of PCa cases | 49 | 43 | 31 | 123 |

* I-PCa= Prostate cancer subgroup of the Middle Eastern population.

** II-PCa= Prostate cancer subgroup of the Caucasian population.

***III-PCa= Prostate cancer subgroup of the Afro-Caribbean population.

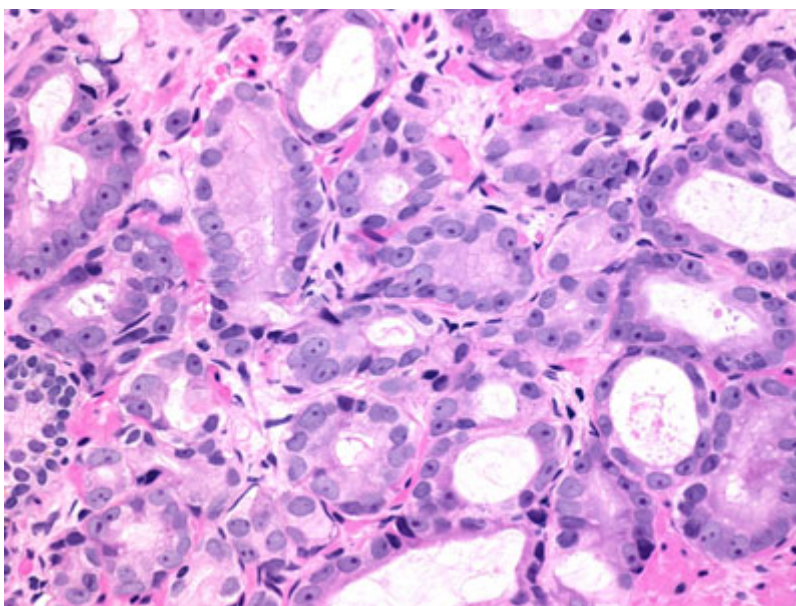


Figure 4-1: Sample of Gleason grade 3+3=6 (x400)

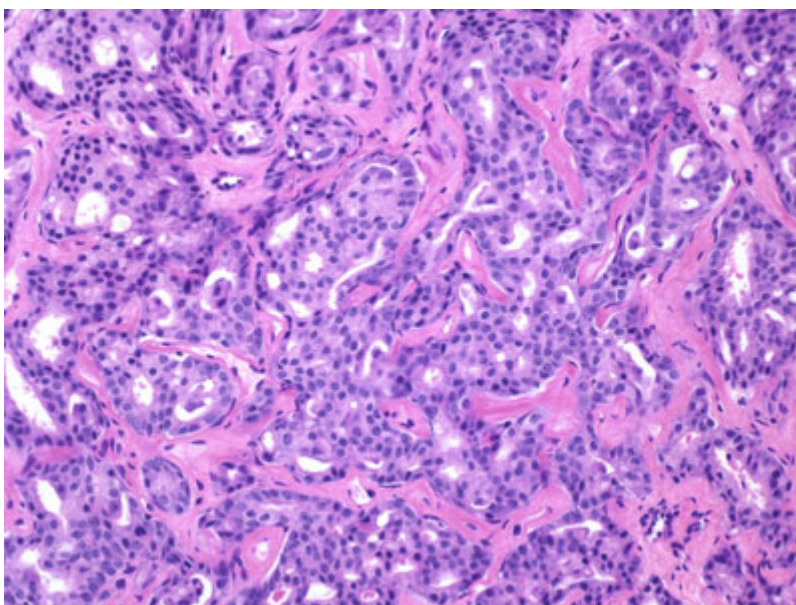


Figure 4-2: Sample of Gleason grade 4+3=7(x200)

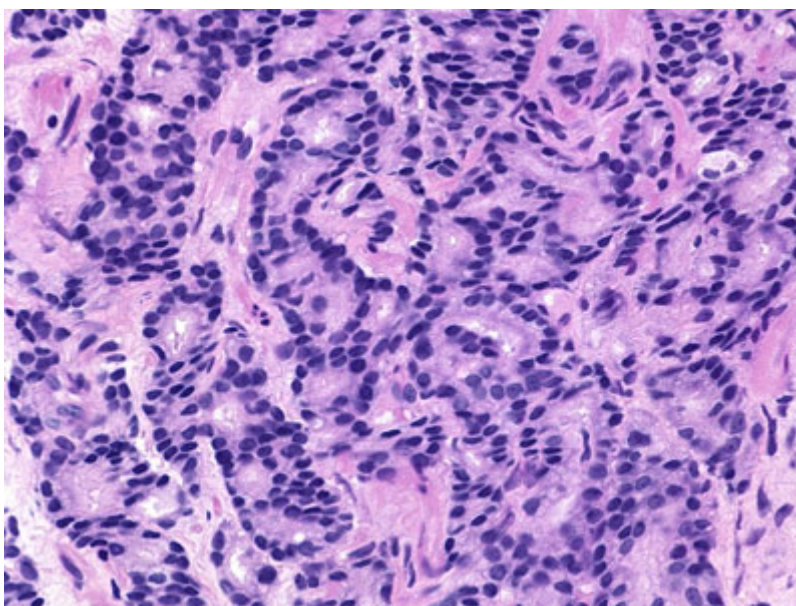


Figure 4-3: Sample of Gleason grade 4+4=8 (x400)

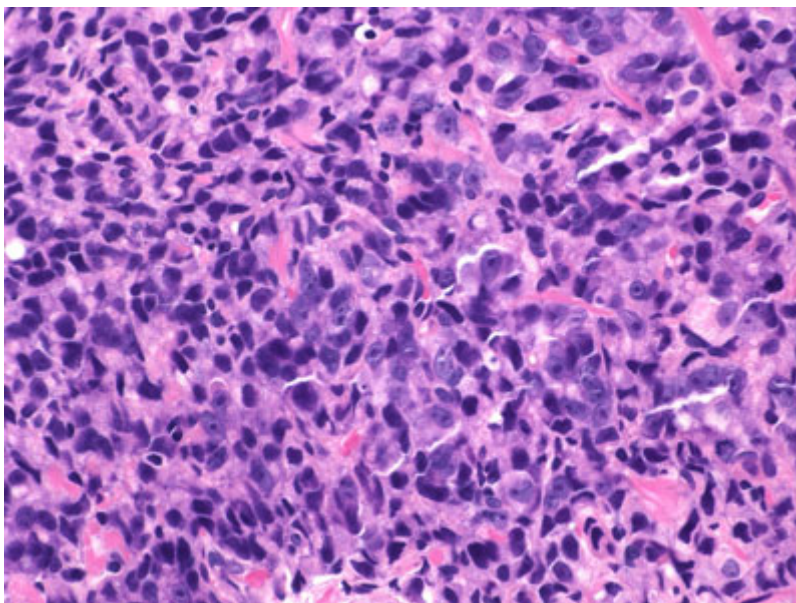


Figure 4-4: Sample of Gleason grade 5+5=10 (x400)

4.2. HPV EXPRESSION IN THE TOTAL STUDY AND CONTROL GROUPS

For the purpose of investigating the HPV presence within the study and control groups using IHC methodology we have used a monoclonal broad spectrum HPV cocktail antibody which reacts with a 56 kilodalton protein present in human HPV subtypes 1, 6, 11, 16, 18 and 31. This novel antibody was raised against the major capsid protein L1 of human papillomavirus types 1, 6, 11, 16, 18 and 31 using a recombinant vaccinia virus that expresses the L1 protein of HPV types 1, 6, 11, 16, 18 and 31 as a target for the immunohistochemical reaction.

The immunohistochemical testing of the total PCa and BPH populations has been optimised firstly on cervical cancer tissue samples known for being positive for HPV infection, then performed on the study and control group samples using diluted 1:150 mouse monoclonal HPV cocktail antibody (ab2417, abcam, UK). Positive controls from cervical cancer samples have been included in all the experiments. The negative controls have been prepared by following the same IHC staining protocol but without the step of adding the primary antibody.

The IHC experiments revealed that 25 cases were positive out of 123 cases in the PCa study group (Figure 4-5), while only 10 cases were positive out of 267 BPH control cases (Table 4-2). These results are statistically significant by applying non-parametric test for independent samples using Mann-Whitney U test for two independent groups (p -value=0.0016).

Table 4-2: HPV status in the study and control groups by IHC using HPV antibody.

| Group | PCa* | BPH** |
|-----------------------|------------|-------------|
| HPV positive by IHC | 25 (20.3%) | 10 (3.7%) |
| HPV negative by IHC | 98 (79.6%) | 257 (96.2%) |
| Total number of cases | 123 | 267 |

*PCa= total prostate cancer population

** BPH = total benign prostatic hyperplasia population.

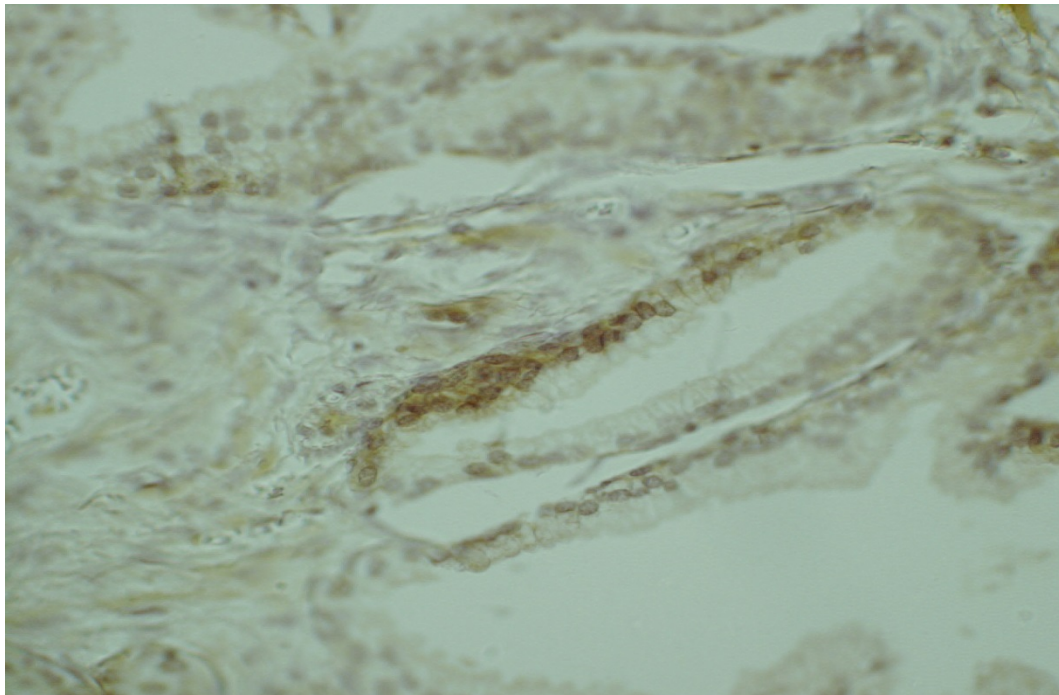


Figure 4-5: Immunostaining of prostate cancer showing HPV positive (x400)

Moreover, the results have shown that all the positive samples for HPV infection by IHC from the PCa and BPH subgroups were also positive by multiplex PCR using SPF1/2 primer set. On the other hand, the results have shown that 18 samples of the 98 negative PCa samples by HPV

cocktail antibody were positive for HPV viral DNA by multiplex PCR using SPF1/2 primers. Likewise, it has been shown that 15 samples of the 247 negative BPH samples from the control groups which have been tested by the HPV cocktail antibody were positive by the same PCR method (Table 4-3).

Table 4-3: Comparison between the negative results of IHC using HPV cocktail antibody and the multiplex PCR positive results using SPF1/2 primer set.

| <i>Group</i> | <i>PCa*</i> | <i>BPH**</i> |
|----------------------------|-------------|--------------|
| <i>HPV-negative by IHC</i> | 98 | 257 |
| <i>HPV-positive by PCR</i> | 18 | 15 |

*PCa= total prostate cancer population.

** BPH = total benign prostatic hyperplasia population.

Given that the PCR test is more definitive in confirming the presence of the HPV viral genome in the tissue sample, it has been used as standard parameter to calculate the sensitivity and specificity of the HPV cocktail antibody IHC test in PCa cases by IHC, which came as 58.1% and 100%, respectively.

In addition, the slides of each specimen in the study and control groups were examined by looking for cytoplasmic/membrane staining. The number of the positively stained cells in four medium power fields (x200) was counted and an average of the number of positive cells was made. The grade of the reaction was quantified using a visual analogue scale as Grade I where 1-10 stained cells per medium power field , Grade II where

11-20 stained cells per medium power field are found, and Grade III where $20 <$ stained cells are found per medium power field.

The study of IHC grading in the total PCa group using HPV cocktail antibody resulted in the frequency of 84%, 16% and 0% for IHC Grade 1 (Figure 4-6), Grade 2 (Figure 4-7) and Grade 3 respectively, while the frequencies of the IHC grading in the total BPH group showed the frequency of 90%, 10% and 0% for IHC Grade 1, Grade 2 and Grade 3 respectively (Table 4-4). These results are statistically significant using Mann-Whitney U test for two independent groups ($p\text{-value} < 0.0001$).

Moreover, the statistical analysis of the distribution of the IHC grading in the total PCa using non-parametric Chi-Square goodness-of-fit test resulted in rejection of the null hypothesis that the IHC grades in PCa group categories happen in equal probabilities ($p < 0.001$). In addition, it has been noted that most of the cases which are positive for HPV infection are at IHC grade 1.

Table 4-4: IHC grading of the positive HPV samples in the total study and control groups.

| <i>Group</i> | <i>PCa*</i> | <i>BPH**</i> |
|------------------------------|-----------------|----------------|
| <i>Grade 1</i> | 21 (84%) | 9 (90%) |
| <i>Grade 2</i> | 4 (16%) | 1 (10%) |
| <i>Grade 3</i> | 0 (0%) | 0 (0%) |
| <i>Total number of cases</i> | 25 | 10 |

*PCa= total prostate cancer population.

** BPH = total benign prostatic hyperplasia population.

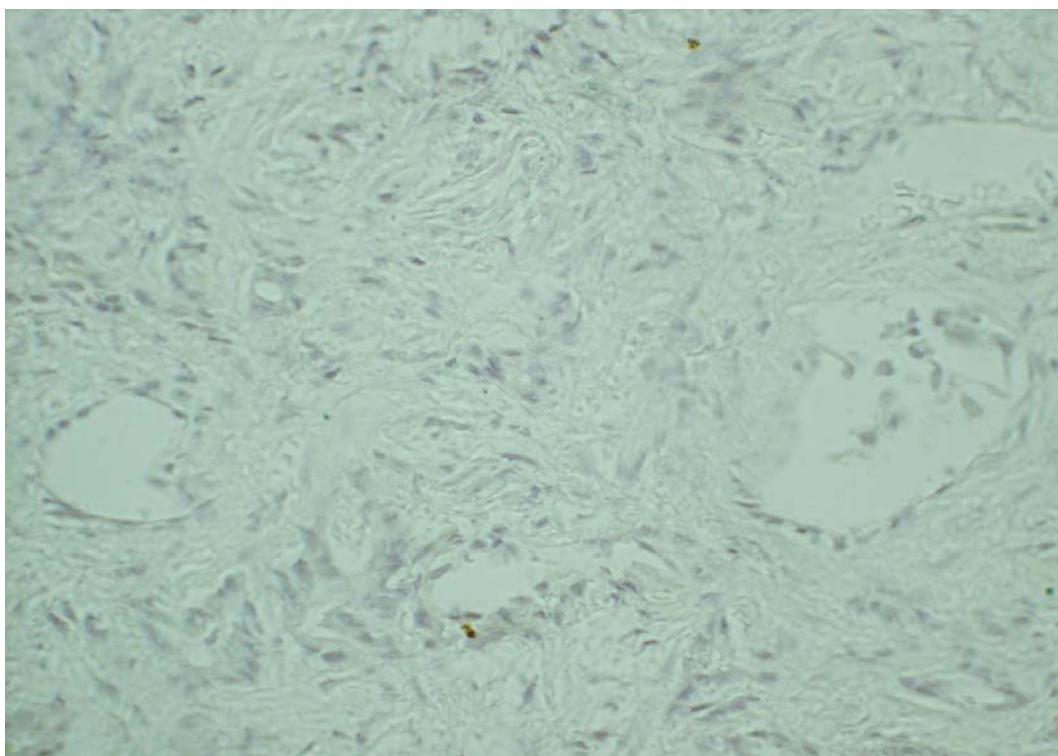


Figure 4-6: Immunostaining of prostate cancer showing HPV positive (grade I) (x200)

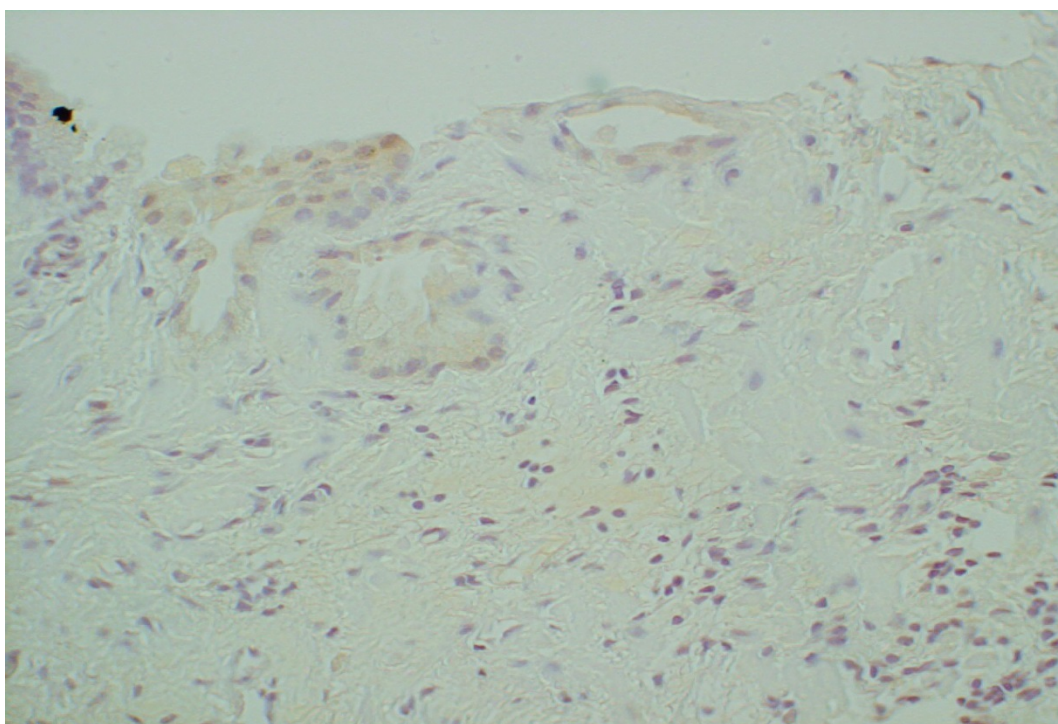


Figure 4-7: Immunostaining of prostate cancer showing HPV positive (grade II) (x200)

4.3. DETECTION OF E6 PROTEIN OF THE HIGH-RISK HPV_s

In the current study, we have designed IHC experiment using anti E6 protein primary antibody (abcam-C1P5), which can detect the high-risk HPV16-E6 as well as the HPV18-E6 proteins in the tissues of the study and control groups. The direct aim of this experiment was to detect if there is a deletion of the L1 ORF in any sample during the process of integration of the high-risk HPV DNA within the host genome. This process can happen as a part of the active phase of infection, which will result in loss of expression of the L1 capsid protein by the integrated HPV DNA but keep expressing the E6 protein if the virus is in active form. Moreover, due to the difference in the sensitivity threshold between PCR and IHC technologies, this experiment might be helpful in differentiating any latent infection by high-risk HPV, which is expressing E6 in several copies per cell but not in an enough number to be detected by IHC, though it would be positive by PCR methods.

Firstly, the immunohistochemical protocol has been optimised firstly on cervical cancer samples known for being positive for HPV-18 infection, before it was performed on the study and control group samples. In this IHC experiment we have used diluted 1:50 mouse monoclonal anti E6 protein (abcam-C1P5). Positive controls from cervical cancer tissues have been included in all the experiments. The negative controls have been prepared by omitting the step of adding the primary antibody.

We found that 8 cases showed positivity for E6 protein by IHC out of 123 PCa cases in the study group. On the other hand, there was no BPH case showing positivity for E6 protein by IHC in the total control group (Table 4-5). It was noted that only few cells were stained positively for E6

protein in all the cases, which showed positivity for E6 protein, which put them all at Grade 1 of IHC staining (Figure 4-8).

Table 4-5: E6 protein status in the total study and control group.

| Group | PCa | BPH |
|-------------------------------------|--------------------|-------------------|
| <i>E6***-positive</i> | 8 (6.5%) | 0 (0%) |
| <i>E6***-negative</i> | 115 (93.5%) | 267 (100%) |
| <i>Total Number of Cases</i> | 123 | 267 |

*PCa= total prostate cancer population.

** BPH = total benign prostatic hyperplasia population.

*** E6 = E6 protein of the high-risk HPVs type 16 and 18.

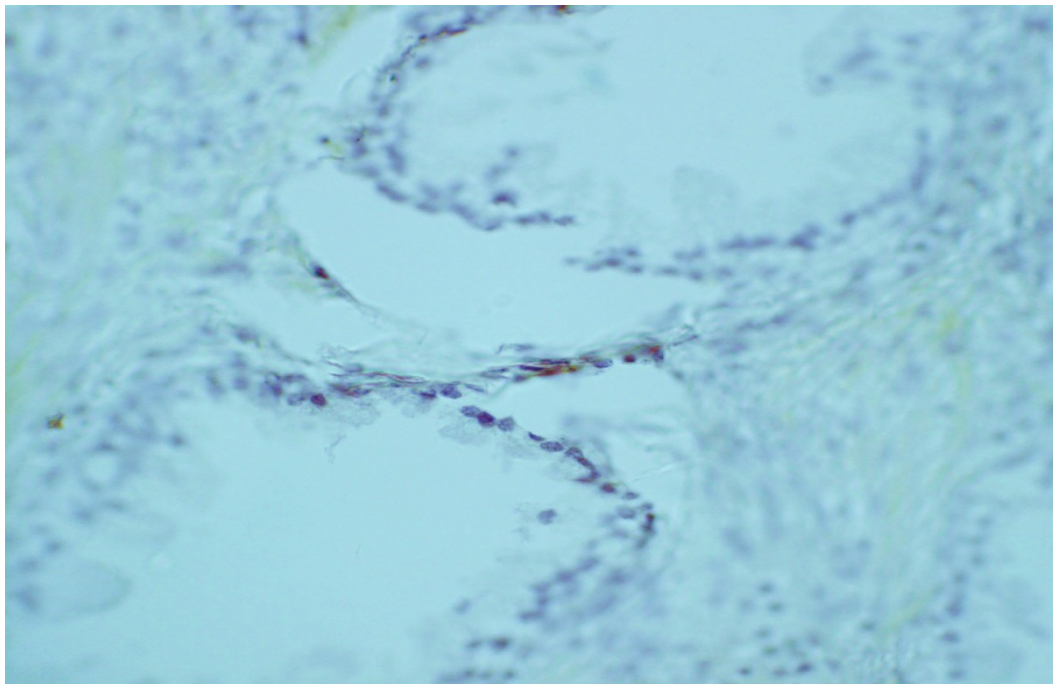


Figure 4-8: Immunostaining of prostate cancer showing HPV E6 positive (grade 1) using HPV 16 and 18 anti E6 protein (abcam-C1P5) (x400)

Moreover, it has been found that out of the 8 cases which showed positive IHC staining for high-risk E6 protein, 3 cases were infected by type 16 of the HPVs, and the rest were infected with type 18 (Table 4-6). In other words, we did not find any case positive for E6 protein but negative for the high-risk HPV infections.

Table 4-6: E6 protein status in the study group using IHC.

| HPV Types | | | | | | |
|----------------------|-------------------------|----------------------|---------------|-----------------------|---------------|--------------------------------------|
| | <i>HPV Negative</i> | <i>Low Risk HPVs</i> | | <i>High Risk HPVs</i> | | <i>Total number of cases</i> |
| | | <i>HPV-6</i> | <i>HPV-11</i> | <i>HPV-16</i> | <i>HPV-18</i> | |
| <i>PCa*</i> | 80 | 5 | 8 | 12 | 18 | 123 |
| <i>E6**-positive</i> | 0 | 0 | 0 | 3 | 5 | 8 |

*PCa= total prostate cancer population.

** E6 = E6 protein of the high-risk HPVs type 16 and 18.

Additionally, we found that the frequencies of HPV high-risk E6 protein within the PCa study sub-groups were 37.5%, 50% and 12.5% for Gleason 6, Gleason 7 and Gleason 8-10 respectively (Table 4-7). The statistical assessment of these results by Chi-Square goodness-of-fit test indicates that there is no association between the high-risk HPV-E6 existence and the grading of the prostate cancer in the total study group ($p= 0.417$).

Table 4-7: The association between the high-risk E6-positive cases and Gleason scoring.

| <i>Group</i> | <i>E6*-positive</i> |
|------------------------------|---------------------|
| <i>Gleason 6</i> | 3 (37.5%) |
| <i>Gleason 7</i> | 4 (50%) |
| <i>Gleason 8-10</i> | 1 (12.5%) |
| <i>Total number of cases</i> | 8 |

* E6 = E6 protein of the high-risk HPVs type 16 and 18.

Likewise, we found that the frequencies of high-risk HPV were 62.5% %, 25%, 12.5% % in the T1-2N0M0, T3-4N0/xM0 and T2-4Nx/+M1 stages in the total study group respectively. The statistical analysis of these results by Chi-Square goodness-of-fit test indicated that there is no association between the high-risk HPV-E6 existence and the staging of the prostate cancer in the total study group ($p= 0.197$).

Table 4-8: association between tumour stage and infection with high-risk HPV.

| <i>TNM Staging</i> | <i>E6*-positive</i> |
|------------------------------------------------------|---------------------|
| <i>Organ-confined disease</i> <i>T1-2N0M0</i> | 5 (62.5%) |
| <i>Locally advanced disease</i> <i>T3-4N0/xM0</i> | 2 (25%) |
| <i>Metastatic</i> <i>T2-4Nx/+M1</i> | 1 (12.5%) |
| <i>Total number of cases</i> | 8 |

* E6 = E6 protein of the high-risk HPVs type 16 and 18.

4.4. THE STATUS OF THE P53 PROTEIN IN THE TOTAL AND STUDY GROUPS

To evaluate the involvement of *p53* inactivation in the development of prostate cancer, we have investigated the status of the *p53* gene in the total study and control groups by IHC method. The immunohistochemical protocol optimisation procedure on breast cancer samples, which are known to be positive for *p53* mutant protein, has been followed, before it was performed on the study and control group samples, and resulted in an optimal dilution of 1:50 for the anti-*p53* primary mouse monoclonal antibody (PAb 240, abcam, UK) which recognizes mutant *p53* proteins but not wild type *p53* protein in its native form [339]. Positive controls from breast cancer cases have been included in all the experiments on the study and control groups. The negative controls were prepared by following all the steps stated in the IHC protocol apart from the step of adding the primary antibody.

The IHC experiments have revealed that 29 cases were positive out of 123 cases in the PCa study group (Figure 4-9). Nevertheless, interestingly, there was no case showing positive signal for the *p53* protein out of 267 BPH control (Table 4-9). These results are statistically significant by Mann-Whitney U test for two independent groups ($p\text{-value} < 0.001$).

Table 4-9: P53 protein status in the study and control groups

| Group | PCa* | BPH** |
|--------------------------------------|-------------------|-------------------|
| <i>Anti-P53 positive results</i> | 29 (23.5%) | 0 (0%) |
| <i>Anti-P53 negative results</i> | 94 (76.5%) | 267 (100%) |
| Total number of cases | 123 | 267 |

*PCa= total prostate cancer population.

**BPH= total benign prostatic hyperplasia population

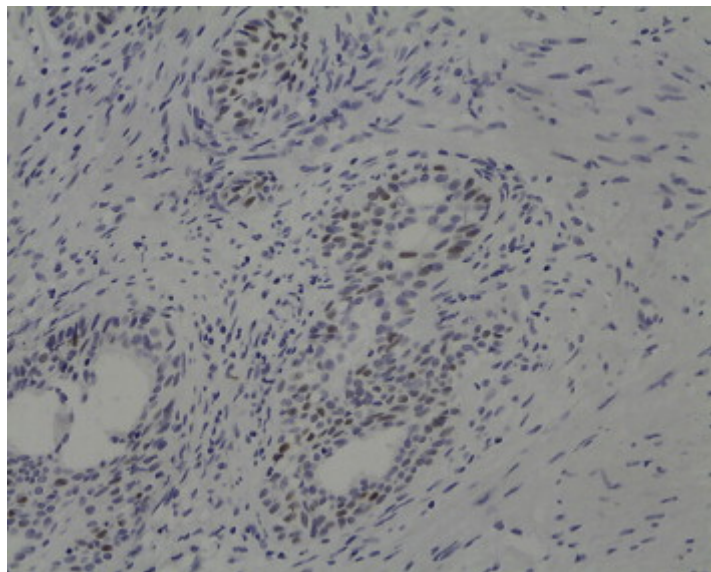


Figure 4-9: Immunostaining of prostate cancer showing P53 positive staining (x200)

Moreover, it has been shown that only 2 cases out of 30 PCa cases (6.6%), which showed positivity to high-risk HPV DNA test by reverse hybridization line probe assay method, were positive for *P53* by IHC staining. In addition, 3 cases of the 13 cases (23%) which showed low-risk HPV infections were *P53* positive. On the other hand, testing the status of

the *P53* gene in the PCa cases, which showed negativity for the high-risk HPV DNA test by the same methods, showed that it was positive in 24 out of 80 cases (30%) in this subgroup. (Table 4-10).

Table 4-10: The association between *p53* status and HPV status in the total PCa study group.

| HPV Types | | | | | | |
|-------------------------|-------------------------|----------------------|---------------|-----------------------|---------------|--------------------------------------|
| | HPV Negative | Low Risk HPVs | | High Risk HPVs | | Total number of cases |
| | | HPV-6 | HPV-11 | HPV-16 | HPV-18 | |
| P53 Positive | 24 | 1 | 2 | 0 | 2 | 29 |
| PCa* | 80 | 5 | 8 | 12 | 18 | 123 |

*PCa= total prostate cancer population.

Furthermore, testing the association between high-risk E6 proteins and the *p53* status within the PCa study group using IHC revealed that when the IHC signal for E6 was positive, there were 12.5% of the cases showing *p53* positive signal and 87.5% negative for *p53*. On the other hand, when there was no IHC signal for E6 protein, there were 24.3% of the cases showing positive signal for the *p53* while the remainder 75.7% were negative for *p53* (Table 4-11). These results are not statistically significant using fisher exact test ($p=0.679$).

Table 4-11: The association between P53 and high-risk E6 protein in the study group using IHC.

| P53 status | High-risk HPV E6 status in PCa* cases | |
|-----------------------|---------------------------------------|---------------|
| | E6** positive | E6** negative |
| P53 positive | 1 (12.5%) | 28 (24.3%) |
| P53 negative | 7 (87.5%) | 87 (75.7%) |
| Total number of cases | 8 | 115 |

*PCa= total prostate cancer population.

** E6 = E6 protein of the high-risk HPVs type 16 and 18.

4.5. THE STATUS OF THE p16^{INK4a} IN THE TOTAL STUDY AND CONTROL GROUPS

To investigate the status of the p16^{INK4a} transcription factor in the study and control groups, we examined all the samples in the study and control groups anonymously by IHC using diluted 1:200 anti- p16^{INK4a} primary antibody (2D9A12, abcam, UK). The dilution and the other IHC protocol steps have been optimised on cervical cancer sections before starting the actual experiments on the study and control groups. Also, positive and negative controls from cervical cancer cases have been included in all the IHC experiments. The negative controls have been prepared by following all the IHC protocol steps apart from the step of adding the primary antibody.

The IHC experiments showed that 24 cases were positive out of 123 cases in the PCa study group (Figure 4-10). Also, it was found that 2 cases out of 267 BPH control cases were positive for the p16^{INK4a} (Table 4-11). These results are statistically significant by non-parametric test for independent samples Mann-Whitney U test for two independent groups (p-value=0.014).

Table 4-12: $p16^{INK4a}$ transcription factor status in the study and control groups.

| Group | PCa* | BPH** |
|------------------------------------------------------------|-------------------|---------------------|
| Anti- $p16^{INK4a}$ positive results | 24 (19.5%) | 2 (0.74%) |
| Anti- the $p16^{INK4a}$ negative results | 99 (80.5%) | 265 (99.26%) |
| Total number of cases | 123 | 267 |

*PCa= total prostate cancer population.

** BPH = total benign prostatic hyperplasia population.

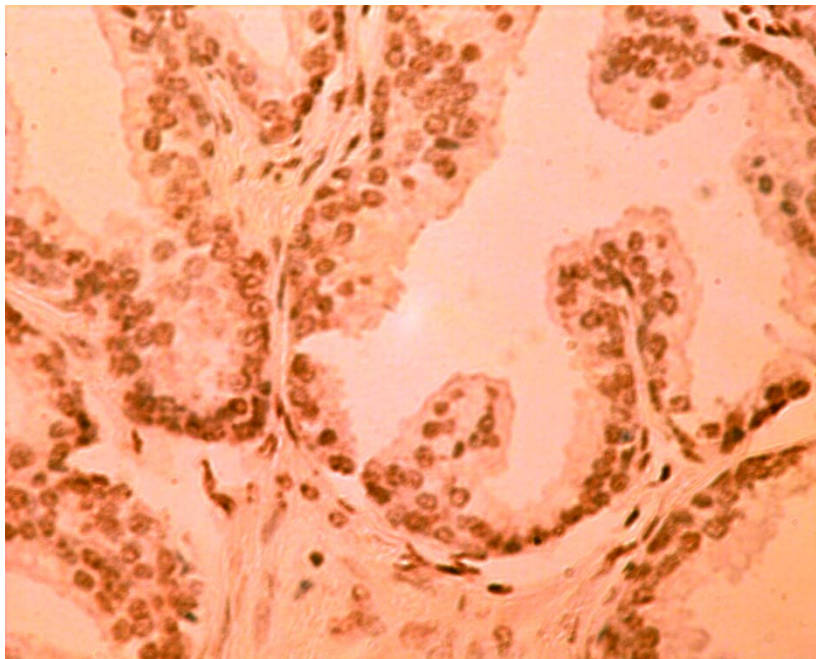


Figure 4-10: Immunostaining of prostate cancer showing $p16^{INK4a}$ positive staining (x400)

Furthermore, it has been found that $p16^{INK4a}$ positive signal could be detected in different frequencies within the HPV positive as well as the HPV negative subgroups in the PCa cases (Table 4-13). Also, it was

notable that the statistical analysis of those results using Chi-Square goodness-of-fit test for one categorical variable showed that there is no association between HPV status and the p16^{INK4a} transcription factor (p=0.688).

Table 4-13: Association between the p16^{INK4a} and the HPV infections status in the study group.

| HPV Types | | | | | | |
|-----------------------------------------|-----------------|---------------|--------|----------------|----------|-----------------------------|
| | HPV Negative | Low Risk HPVs | | High Risk HPVs | | Total number of cases |
| | | HPV-6 | HPV-11 | HPV-16 | HPV-18 | |
| <i>p16^{INK4a} Positive</i> | 13(16.2%) | 1(20%) | 2(25%) | 3(25%) | 5(27.7%) | 24(19.5%) |
| <i>PCa*</i> | 80 | 5 | 8 | 12 | 18 | 123 |

*PCa= prostate cancer population.

Moreover, it has been found that 1 of the 2 BPH cases, which showed positivity for p16^{INK4a} transcription factor by IHC, was positive for high-risk HPV DNA. However, the other BPH case was negative for high-risk HPV DNA (Table 4-14).

Table 4-14: Association between the *p16INK4a* and the HPV infections status in the control group.

| HPV Types | | | | | | |
|-------------------------------------|-------------------------|----------------------|---------------|-----------------------|---------------|--------------------------------------|
| | HPV Negative | Low Risk HPVs | | High Risk HPVs | | Total number of cases |
| | | HPV-6 | HPV-11 | HPV-16 | HPV-18 | |
| <i>p16INK4a</i> Positive | 1(0.4%) | 0 | 0 | 1(11%) | 0 | 2 (0.7%) |
| BPH* | 242 | 4 | 5 | 9 | 7 | 267 |

*BPH = total benign prostatic hyperplasia population.

4.6. *in situ* TUNEL APOPTOSIS ASSAY

TUNEL *in situ* assay experiments were carried out according to the manufacturer instructions (Promega DeadEnd apoptosis detection system), using 5µg/ml proteinase k incubation for 5 minutes. A positive control slide was included in each run according to the protocol, and a negative control slide without adding TUNEL reaction mixture was included.

For TUNEL scoring, the average number of positive nuclei was calculated in 10 high-power microscopic fields (×400) selected from a central region of the tumour areas avoiding areas containing necrosis. TUNEL technique is based on the specific binding of TdT to 3'-OH of DNA fragments in apoptotic nuclei, permitting the *in situ* visualisation of apoptotic cells (Figure 4-11). However, a very careful approach has been followed when

evaluating the morphologic results as necrotic cells and some inflammatory cells, including neutrophils and lymphocytes, are stained sometimes.

Due to the small amount of tissues remained in the paraffin blocks we were only able to do the TUNEL *in situ* assay on 42 samples of the total study groups of 123 PCa cases. The TUNEL *in situ* assay score results obtained from this PCa study group along with the E6 status were summarised in (Table 4-15).

Statistical analysis using Chai-Square goodness-of-fit test showed that these results are not statistically significant ($p=1$). Thus, these results showed that there is no direct association between the presence of E6 of the high-risk HPV infections and the extent of apoptosis in the PCa total study group.

Table 4-15: Association between TUNEL *in situ* assay score and high-risk E6 protein of the HPVs in the PCa study group.

| High-risk E6 protein | Number of samples | TUNEL <i>in situ</i> assay score median (interquartile rang) |
|----------------------|-------------------|--------------------------------------------------------------|
| E6-positive | 5 | 5(2-10) |
| E6-negative | 37 | 5(2-11) |

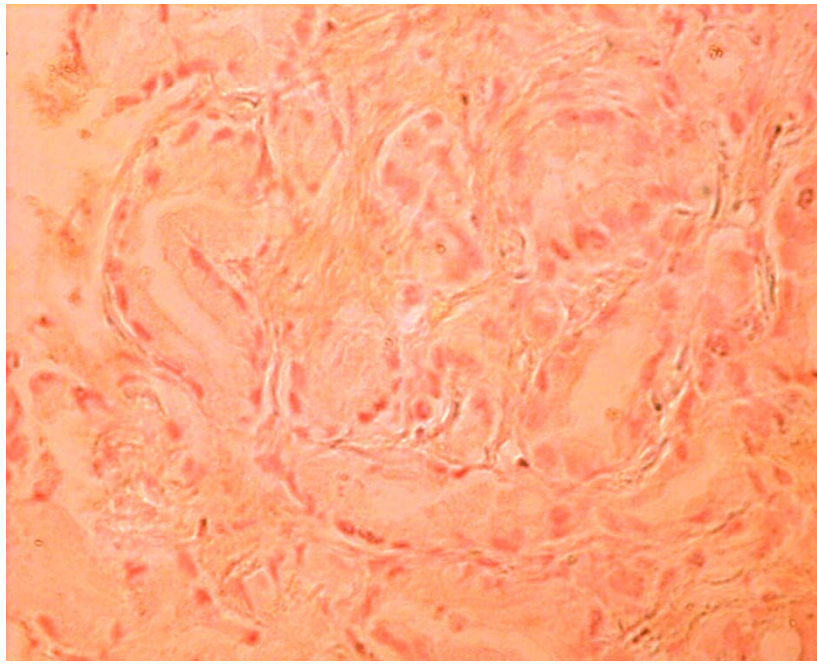


Figure 4-11: TUNEL in situ assay on prostate cancer sample showing the apoptotic cells as black small dots (x400)

4.7. DISCUSSION

When Gleason compared his grading system with survival rates, it was noted that in tumours with two distinct tumour patterns, the observed number of deaths generally fell between the numbers expected based on the primary pattern and that based on the secondary pattern [267]. Because both the primary and the secondary patterns were influential in predicting prognosis, there resulted a Gleason sum obtained by the addition of the primary and secondary grades. If a tumour had only one histological pattern, then for uniformity the primary and secondary patterns were given the same grade. Gleason sums range from 2 ($1 + 1 = 2$), which represents tumours uniformly composed of Gleason pattern 1 tumour, to 10 ($5 + 5 = 10$), which represents totally undifferentiated

tumours [44]. Synonyms for Gleason sum are combined Gleason grade and Gleason score [44]. Pathologists may assign only a Gleason pattern rather than a Gleason sum in cases with limited adenocarcinoma of the prostate on needle biopsy [340]. However, it should be made perfectly clear by the pathologist that she or he is assigning only a pattern and not a sum. Cases in which the pathologist signs out a case as Gleason grade 4 (i.e., Gleason pattern 4) may be misconstrued as Gleason sum 4. Consequently, for the purpose of the current research it has been agreed to assign both a primary and a secondary pattern even when presented with limited cancer so as to not give rise to any confusion. The Gleason system does not account for the existence of a tertiary (third most prevalent) pattern [341]. In radical prostatectomy specimens, it has been demonstrated that tertiary high-grade components adversely affect biologic behaviour [342]. It is proposed that the Gleason system for radical prostatectomy specimens be modified, either by recording the Gleason grade as a combination of the most common pattern along with the highest grade pattern or by giving the routine Gleason grade (primary and secondary patterns) with a note stating that there is a tertiary high-grade pattern [343].

One of the aims of this research was to test the reliability of a monoclonal broad spectrum HPV antibody, which was raised against the major capsid protein L1 of human papillomavirus types 1, 6, 11, 16, 18 and 31. There is a big debate about the usefulness, sensitivity and specificity of different IHC antibodies as a detecting tool for the HPV infections. This debate is even more pronounced when paraffin-embedded tissues are used. In the present study, the results obtained were compared with the results of the multiplex PCR using SPF1/2 primer set to determine reliability of this antibody in detecting HPV L1 protein in paraffin-embedded malignant

and benign prostate samples in terms of the sensitivity, specificity of this IHC test.

The modest sensitivity of this HPV cocktail antibody test, which was 58.1%, tells how likely this test is to be positive in samples which are confirmed to have HPV viral DNA by multiplex PCR. This means that this test would not be suitable candidate for being a screening test, which can precede the other conventional molecular tests, which rely on detecting the viral DNA like PCR and hybrid capture II. On the other hand, the specificity of this test, which came as 100%, tells how likely the test is to come back negative in a sample which is known to be negative for HPV viral DNA by multiplex PCR. This means that IHC test would be a very suitable candidate as complementary test after the initial confirmation has been made by another method. The latter conclusion seems to put the HPV IHC test in the middle between the Pap test which usually shows variable (poor to moderate) sensitivities (30 to 87%) [344], when equivocal cases need to be repeated to improve diagnostics, and the other chromatographic hybridisation technologies like hybrid capture II, which has higher sensitivities up to 85% [345, 346].

However, the proportion of both HPV-positive in malignant and benign specimens, as would be detected by a purely qualitative assay like IHC or PCR, strongly depends on the sensitivity of the respective assay. Decreasing the limit of detection would increase the number of positive samples detected and *vice versa*. Therefore, our data offer direct experimental evidence of the notion that different assay sensitivities of the various molecular tests are one reason for the contradictory results reported thus far.

The E6 protein of high-risk HPVs is classified as an oncoprotein and can transform human mammary cells and cooperate with E7 in transforming human epithelial cells [106]. E6 is expressed early after viral infection and facilitates viral production by conferring several cellular changes and by prolonging cellular lifespan by disrupting the *p53* role as tumour suppressor gene [150]. To achieve the latter effect high-risk HPV E6 interacts with a ubiquitin ligase, called the E6 associated protein (E6-AP), and forms complexes with *p53* protein, which is then degraded through ubiquitin dependent mechanisms [187, 208]. Moreover, E6 can block translocation of *p53* into the nucleus and thereby inhibit the gene regulatory functions of *p53* [347].

Only a few systematic studies exist on the expression of E6 protein of the oncogenic HPV types in prostate tissues. But, there are few epidemiological data, in addition to cell culture experiments, indicate a possible role of HPVs for the pathogenesis of prostate tumours [300, 348]. However, this subject remains controversial, in large part due to contradictory results regarding the detection of the viral nucleic acids within prostate cancer [300].

In our study (E6) has been found in 6.5% of the total PCa study group. This result supports conclusions drawn from earlier investigations on the presence of HPV16 and HPV18 DNA in prostate specimens [278, 349].

In principal, the low numbers of E6 oncoproteins detected by IHC in a subset of prostate tissue specimens have been reflected in the fact that all the E6-positive samples were at IHC Grade 1. Therefore, it can be proposed that the cells bearing the virus are either present within the sample in a very low frequency, or that only a fraction of the cells is carrying the viral DNA.

In addition, it has been reported that the deletion of L1 ORF of the high-risk HPVs might be a common phenomenon during the active integration phase of HPV infections in cervical cancer which might be up to 41% [274]. In these circumstances it is very common to have negative amplification signal by testing these cases by PCR method using primers targeting the L1 ORF, alongside a parallel positive signal for the E6 protein in a considerable proportion of the samples which do have integrated DNA but deleted L1 ORF region. However, in our study we did not find any results as such, which may indicate L1 deletion is not common in high-risk HPV infections in prostate cancer cases and high-risk HPV viral DNA integration prevalence might not be so frequent .

Similarly, it has been reported that because L1 capsid protein is also the major target of cellular immune response and its loss at early stages of the transformation process may lead to ineffective stimulation of immune responses. Therefore, lack of L1 capsid protein may occur to reduce the cellular immune responses, thereby promoting further transformation of immature epithelial cells. However, we did not find an evidence, which is in line with this hypothesis in our study group as all the cases which showed E6 positive signal by IHC actually did have positive amplification signal for the L1 region of the high-risk viruses by multiplex PCR.

Furthermore, it is well known that DNA tests, such as PCR cannot differentiate among latent, subclinical, and clinically relevant infections. Also, it has been suggested that the normal immune system suppresses latent and clinical HPV cervical infections and that the efficiency of suppression may be HPV type-specific [350]. According to the latter context of HPV latency, there would be low copy numbers of the virus which are enough for the maintenance of the viral life including the capsid

proteins which are encoded by the L1 ORF, but not in an enough copy number to get the E6 protein detectable by IHC method. This hypothesis is concordant with our observation, and might explain why only 26.6% of the total number of PCa cases, which had high-risk HPV infections, expressed E6 protein.

Infections with HPVs can lead to inactivation of the *p53* tumour suppressor gene product by binding to the HPV encoded E6 protein, which leads to a rapid degradation of *p53* through the ubiquitin pathway. The latter event has been considered as an important pathway for malignant progress in HPV-infected cells [351]. In contrast, mutations of the *p53* gene have been found in HPV-negative cervical carcinoma cells [187]. Also, the current dogma in cancer biology is that *p53* alterations to be found in every tumour type, and mostly result from point mutations. However, *p53* gene mutations are not limited to a late event in prostate cancer progression. They can occur early in cancer development and it is now generally accepted that mutations of *p53* occur infrequently in early invasive carcinomas [352]. However, the limitation of current sampling techniques and technology prohibits an accurate determination of the *p53* status in early stage disease [353]. In addition, *p53* is commonly mutated in advanced stages of prostate cancer, as well as in recurrent and metastatic disease, as inferred by immunocytochemical demonstration of accumulated protein or by direct mutational analysis [354]. It is also known that *p53* mutations are associated with increased micro-vessels density, androgen-independence, and metastasis in PCa, raising the possibility that tumourigenesis and acquisition of metastatic ability occur simultaneously, and not sequentially, in PCa cases [355, 356].

The results of our study revealed that *p53* expression is also not related to an HPV infection. In fact, there were HPV-negative cases in our study with *p53* expression. Also, based on the cervical carcinoma model in which mutations in *p53* are very rare and high-risk HPV infections are very frequent in primary tumours in over 90% of the cervical cancer cases, which strengthens the view that *p53* inactivation by high-risk HPVs proteins plays a major role in the pathogenesis of cervical cancer, we looked for any association between the high-risk HPV infections and the *P53* status. We found that *P53* mutant protein was detectable with and without concurrent high-risk HPV, and interestingly no mutant *p53* protein was detected in the BPH cases. Moreover, our results showed that *p53* protein expression happens less frequently when the E6 oncoprotein of the high-risk type 16 and 18 HPVs are detectable in the same PCa samples, which may suggest that the *p53* gene mutation or the presence of the high-risk HPVs oncogenic protein E6 could be involved in the development of prostate cancer, also, high-risk HPVs could participate in *P53* inactivation by E6 in prostate cancer cases. Nonetheless, such a causative relationship need more work to confirm as other causes might be responsible for the low incidence of *P53* mutation in the samples other than the effects of being infected with high-risk HPVs. One of these causes is the loss of chromosome 17p, which occur in advanced stages of prostate cancer and metastatic disease [357], deleting a region that includes the *p53* locus [358]. In addition, it is well known that the frequency of *p53* mutations seems generally to be lower in prostate cancer than in other cancers [359]. A relatively minor role for *p53* in prostate carcinogenesis is consistent with the observation that Li-Fraumeni patients carrying germline *p53* mutations have a low incidence of prostate cancer [360].

The E7 protein of the high-risk HPVs binds and phosphorylates the tumour suppressor *pRb* and inhibits its binding to E2F. Released E2F transcriptional factor stimulates p16INK4a transcription, leading to p16INK4a overexpression, which is also caused by the loss of the negative feedback from free *pRb*, disrupted by HPV E7 [361]. Moreover, overexpression of p16INK4a is known to be observed in cancers of the uterine cervix [362], and is associated in the majority of the cases in this group of cancers with HPV infections [363].

A comprehensive review of the literature reveals that to the best of our knowledge there are no studies, which have simultaneously analysed HPV status and p16INK4a in PCa or BPH cases. Nevertheless, only a few studies have analysed the correlation between the p16INK4a expression and HPV infections in the female genital tract [364].

In the current investigation, which is the first to evaluate simultaneously HPV status and p16INK4a immunoreactivity in these prostatic pathologies, we observed that 22 of 30 the high-risk positive prostatic neoplasms failed to show the type of diffuse strong p16INK4a staining that one would expect to see in HPV-positive tumours based on the observation of HPV-positive cervical neoplasms [365]. Moreover, we found p16INK4a immunoreactivity in 13 cases of the of the total 80 HPV-negative cases of PCa cases. We also detected few cases where p16INK4a was strongly expressed and no HPV was detected. One explanation for our observations could be because the levels of HPV were too low for detection by IHC method or the HPV types may be present but are not amplified by the SPF1/2 system. In addition, this study is consistent with the hypothesis that p16INK4a overexpression in the female genital tract and cervical cancer namely might not be related to HPV carcinogenesis

effect [366]. The latter conclusion contradicts with another report, which found that p16INK4a overexpression is a very useful surrogate biomarker to detect high-risk HPV infections in cervical cancer cases [363]. Nevertheless, the results of our study revealed that p16INK4a overexpression is also not related to an HPV infection. In fact, many cases in our study with p16INK4a overexpression were HPV-negative. Therefore, it can be concluded that in the absence of HPV infection, mutations in the *Rb* gene or E2F transcription factor gene appear to be playing a role in prostate cancer tumourigenesis, and p16INK4a is not a suitable candidate to act as high-risk HPV surrogate marker in prostate pathologies.

On the other hand, few reports argued that hit and run oncogenesis might be involved in prostate cancer and other cancers [180] which may explain, if true, why p16INK4a overexpression could be found in high-risk negative samples. According to this hypothesis the oncogenic virus may infect its target tissue, trigger a misdirected immune response, mutagenic activity, or a permanent chromatin reorganization, which in turn activates oncogenes or silences tumour suppressor and DNA repair genes. Thereafter, cells may become malignant and remain so, even if virus-triggered recombinogenic activities lead to the secondary loss of the virus [180]. For hit and run-mechanisms one would consider a transient, but regular presence of viral genomes or parts thereof at an early stage of the respective tumour [367]. Also, to conclusively prove hit and run-oncogenesis, a complete chain of evidence from epidemiology, histology, serology and molecular biology is required [179]. However, the results from our current retrospective case-control study cannot at its own affirm or reject the hit and run hypothesis concerning HPV infections. So, it might be proposed that a comprehensive prospective cohort study might be essential to assess if the high-risk HPV hit and run oncogenesis is

actually involved in prostate neoplasia, which mainly should rely on the novel high throughput sequencing methods that allow the identification of viral genomes or parts thereof in pre-dysplastic tissue and in early stages of neoplastic development [180].

Repair of damaged DNA is the only natural way to ensure survival in unicellular organisms. Repair, growth arrest, and apoptosis at present can be considered a response to DNA damage in human tissues, although their fate in many cases depends on cell type, location, environment, and extent of damage [368]. Therefore, alterations either in the machinery that senses DNA damage or in the mechanisms that implement a response to DNA damage are considered important in predisposition to cancer [369, 370]. There are two main checkpoints in the control of cell cycle progression: G1/S prior to the replication process of DNA, and G2/M preceding the process of mitosis [371]. The *p53* gene is activated as a response to DNA damage, followed by cell cycle arrest and activation of the apoptosis cascade [372]. Activation of *p53* is carried out through a phosphorylation process that regulates its DNA-binding affinity [373]. One of the main observations about the interaction between the high-risk HPVs protein E6 and the functionality of the *p53* gene in anogenital cancers [374] and skin cancers [375] indicate that the high-risk E6 protein interacts with a ubiquitin ligase, called the E6 associated protein (E6-AP), and forms complexes with *p53* protein, which is then degraded through ubiquitin dependent mechanisms [89], in addition to blocking the translocation of *p53* into the nucleus and thereby inhibit the gene regulatory functions of *p53* [187]. If the latter effects of the high-risk HPV are substantial in prostate cancer they would result in lower levels of apoptosis index within the samples which are positive for the high-risk E6 protein. However, in the current study, no apparent association could be

established between the presence of HPV-DNA or the high-risk E6 protein and apoptosis indices, suggesting that HPV itself is not directly involved in the apoptosis regulatory pathways in prostate cancer. Our results are in agreement with another report, which could not establish any correlation between the high-risk HPV presence and the apoptosis indices in paraffin embedded cervical cancer samples [376]. In contrast with the latter report, a recent study has confirmed that high-risk E6 protein are capable of arresting apoptosis in head and neck HPV-positive cancer cell lines [377]. In conclusion, there is a need for further studies on prostate cancer cell lines after being transfected with E6 DNA to confirm our finding in paraffin embedded samples.

CHAPTER 5: GENERAL DISCUSSION

Prostate cancer is one of the most common malignancies in the Western world. In 2008, the number of new cases in the 27 countries of the European Union was 338,730 (25% of all new cancers) and the number of deaths reached 70,820 (10% of deaths from cancer) [1].

Small foci of PCa are harboured by the prostates in 80% of men over 80 years old [378], and many of these patients die from other causes without knowing they suffer from PCa [379]. In fact, clinically significant PCa generally affects men in their sixties but is probably initiated years or decades earlier; therefore, it is difficult to establish the primary insult that triggers prostate carcinogenesis [380]. Causative factors have been found in other genitourinary malignancies, which can be targeted, like smoking in cases of bladder cancer [381] and the human papillomavirus in cases of cervical cancer [88]. However, the principal risk factors in PCa are not modifiable, which are mainly related to patients' genetic makeup such as African-American origin or having a strong history PCa running in the family [382]. In addition, after the introduction of PSA screening in the early 1990s, PCa incidence has drastically increased along with a shift to earlier stages among newly diagnosed cases [382]. Even if PCa is successfully managed, which is the usual outcome in localised disease, treatment may be accompanied by functional consequences like; erectile dysfunction and incontinence [383]. These side effects impair deeply the patient's quality of life. In addition, there are limited therapeutic options available for advanced PCa, and metastatic disease remains incurable. Finally, the management of PCa within a large population of elderly men imposes an economic burden on health care systems, particularly in the gradually aging Western populations. Finding a potentially modifiable or

preventable risk factor in PCa, such as HPV infection of the genitourinary tract, would be of great medical importance.

It has been suggested that PCa is associated with septic or aseptic chronic inflammation, the source of which may be prostatic infection and other mechanisms [384]. Also, it has been reported that patients with a history of prostatitis were more likely to develop PCa [385]. However, a large population-based case-control study did disagree with the latter conclusion [386].

HPV has been the most extensively investigated pathogen in studies to determine an infectious cause of PCa cases because it has been established as the main etiologic factor in cervical carcinoma and several other anogenital malignancies. The first report of investigating of HPV viral DNA in prostate cancer tissues was in 1990 using PCR. However, this report failed to establish a significant difference between PCa patients and a group of BPH patients [277]. The promising yields of HPV detection and the indecisive conclusion of that study encouraged other researchers to conduct more research on the same subject.

Twenty nine tissue-based studies from 1980 to December 2012 [212, 213, 217, 278-280, 283, 284, 288, 289, 299, 300, 313, 349, 387-401] were identified for the purpose of this study, 22 of which are case-control studies, whereas the remaining 7 papers only analysed PCa samples. The current study was different from all the previous studies in assessing the effects of HPV infection on *P53* and *Rb* genes as well the apoptosis rate within the samples without limiting the scope of the research to detect the viral DNA in the study and control groups. On the other hand, the current study is to the best of our knowledge, the only study that tested the hypothesis of HPV infection in three different ethnic groups at the same time and by the same methodologies.

Moreover, the earlier studies previously covered the same subject of the current research were not homogenous. The diversity of the respective study designs and methods; the sample size which ranged from 17 subjects to over 300 as in the current study; the varied definition of controls as either BPH or healthy subjects; scope of HPV types detected which were usually 16 and 18; detection methods which were PCR in the majority of studies; primers used which mostly targeted L1 or E6/E7 or other gene sequences; sample harvesting, which varied amongst open surgery, transurethral and transrectal approaches; and processing and storage conditions of the prostate tissues as formalin-fixed, paraffin-embedded or frozen samples.

Statistically significant differences between PCa patients and a control group have been established in 4 publications out of the 29 studies earlier mentioned. In one of the early studies [388], HPV DNA of different HPV types was detected in the prostates of 28 out of 68 Japanese PCa patients, whereas all the BPH controls were HPV-free. In addition, the detection rate in this study was correlated with the disease stage and Gleason score, which contradicts with the results of our current study. Another group in Germany used competitive quantitative PCR to detect HPV 16/E6 DNA sequences. One to two copies of viral DNA were detected in most of the benign and malignant frozen samples that were obtained via radical retropubic prostatectomy. Thus, some degree of positivity was observed in most of the samples. With a cut-off value of 300 copies, 21% of the PCa samples were HPV 16 positive ($P = 0.02$) vs. 3% of the BPH controls [300]. Another group in Argentina detected different HPV types in 42% of PCa transrectal biopsy samples, whereas all the BPH specimens were negative ($P < 0.0001$) [213]. It is interesting to note that both studies employed surgical microdissection to maximize the number of tumour cells in the

sample. In contrast, several other trials yielded null results using the microdissection technique [217, 279, 299]. It was notable that PCa patients from Northwestern Mexico were 4 times more likely to harbour HPV DNA in their prostates than their BPH counterparts ($P = 0.027$) [313]. Nevertheless, none of the remaining 24 studies from other countries demonstrated any differences in the presence of HPV DNA between PCa patients and noncancerous controls.

The following potential limitations of laboratory studies that are based on the direct detection of pathogens in prostatic tissue have been proposed: (1) they are restricted to hypotheses that involve persistent prostatic infections; therefore, if a pathogen interacts with the tissue using a hit-and-run mechanism, it may not be detected at the time of analysis; (2) they are usually small in size; (3) they are prone to some selection bias; (4) they do not allow for the assessment of temporal relationships between infection and cancer; (5) the results vary depending on the specific genetic sequences being investigated; (6) the results may vary depending on the amount and location of the tissue sampled, including the possibility of missing a focal infection; and (7) specimens may be contaminated during collection and processing [402].

One specific drawback of large epidemiologic studies of PCa is the definition of the control group. The latent existence of clinically unapparent lesions is an inherent characteristic of PCa. If controls are only defined as “cancer-free” or “PCa-free,” men with subclinical undiagnosed lesions may be included in the control group, which would shift the statistics towards the null [2].

After several years of HPV-DNA analysis in benign and malignant prostate samples, the causal involvement of HPV in prostate carcinogenesis is still a matter of controversial debate. However, the association of HPV with prostate cancer, if substantiated, would be unexpectedly good news for cancer prevention prospects. The discrepant results and methodological insufficiency of these analyses have already been discussed in by few researchers [212, 279, 281, 348, 403-407]. It has been speculated that, due to the detection of HPV-DNA in urethral [408] and anal [409] tissues, the discrepancies are probably as a result of HPV contamination from nearby tissues during the sampling procedure. Based on these data some authors have recommended radical prostatectomy as the tissue source, as well as an exhaustive microdissection of the neoplastic sample [279]. We propose microdissection of the neoplastic samples to exclude contaminating skin tissue and minimize stromal content from the samples.

Another reason for the conflicting results might be in line with the researches, which reported that in cervical cancer there were cases of persistent infection without production of HPV viral particles, which is usually attributed to the viral genomes in the long-living basal epithelial cells, which do not express L1 Cpasid protein. Thus, loss of L1 Cpasid protein expression may be the case in two statuses of viral DNA; one is the integration of viral DNA into the host genome with the loss of the L1 region and the other is latent infection with low or no synthesis of HPV oncoprotein and no HPV production.

Some other recent studies [289, 392] have also observed no associations between prostate cancer and HPV. The proposed reason for these observations was mainly that prostate cancer does not meet some of the expected features of HPV-caused cancers, as prostate cancer is not

squamous cell in origin and does not occur at anatomic sites of exposure by direct contact [410].

Our data give additional evidence to explain some of the previously reported contradictory findings regarding the prevalence of viral DNA in prostate cancer and BPH specimens. However, to further analyse the potential role of oncogenic HPVs in a subset of prostate cancers, additional quantitative investigations, aiming at the detection of viral DNA on the cellular level, will be necessary.

In our view, these discrepancies, observed by different researchers, could be due to different methodologies used, different interpretation criteria, and possibly other technical aspects.

In this study, we investigated the question of whether the presence of HPV DNA in the tumours samples, and staging of prostatic neoplasms could be considered as prognostic factors. Our investigation and analysis revealed that the survival of these tumours seems to be independent of HPV status.

Moreover, compromised immunity during the course of prostate cancer development might be responsible for reactivation of a latent HPV infection due to the cancer cachexia and might not be responsible for causing prostate cancer.

Further research in the field of PCa and infections may bring new insights into the matter, especially if modern laboratory techniques are used, as a definitive discovery of an infectious agent like the HPV that is directly

associated with PCa could have significant therapeutic implications for millions of patients in the UK and worldwide [378].

APPENDICES

APPENDIX 1: Data extraction sheet

Information to be extracted from patients' notes

PATIENT DETAILS:

Patient code:

DOB:

Place of birth:

Ethnic Origin (delete as appropriate): White / African-Caribbean/ Middle Eastern

CLINICAL HISTORY

Previous prostate problems? Yes/No delete as appropriate

If yes:

Mode of presentation:

1. GP referral
2. Inpatient referral
3. Emergency
4. Incidental
5. High PSA Level
6. Others (please specify):

Clinical presentations:

1. Lower urinary tract symptoms
 - Acute urinary retention
 - Nocturia

- Frequency
 - Dysuria
 - Hesitancy
 - Urgency
 - Poor stream
 - Haematuria
 - Other (please specify)
2. Urinary tract infection
 3. Sexually transmitted diseases
 4. Weight loss
 5. None of the above

Family history of prostatic cancer?

Yes/No delete as appropriate

Past medical history:

1. Diabetes
2. Hypertension
3. Cardiac
4. Others (please specify):

Prostate cancer?

Yes/No delete as appropriate

If yes:

Rectal examination findings:

1. Normal prostate

2. Enlarged benign prostate
3. Clinically suspicious prostate

Clinical stage:

Investigations (please circle appropriate response)

- | | | |
|-----------------------------|-----|----|
| 1. TRUS | YES | NO |
| 2. CXR | YES | NO |
| 3. PSA | YES | NO |
| 4. BONE SCAN | YES | NO |
| 5. CT SCAN | YES | NO |
| 6. MRI | YES | NO |
| 7. OTHERS (please specify): | | |

APPENDIX 2: Table showing the tumour grading, staging, PSA and survival for each individual prostate cancer case which showed positivity for the high-risk HPV infections, along with the P53 codon 72 polymorphism, viral load, P53, P16 and E6 protein statuses.

| Case | Gleason Grading | Staging | PSA (µg/L) | Survival (months) | P53 codon 72 polymorphism | Viral Load ng/µl | E6 | P53 | p16 ^{INK4a} |
|------|-----------------|------------|------------|-------------------|---------------------------|------------------|-----|-----|----------------------|
| 1 | 7 | T1-2N0M0 | 5.2 | 36 | Arg/ Arg | 1.21 | Neg | Neg | Neg |
| 2 | 6 | T3-4N0/xM0 | 7.1 | 49 | Pro/ Arg | 1.37 | Neg | Neg | Pos |
| 3 | 7 | T2-4Nx/+M1 | 7.8 | 34 | Arg/ Arg | 0.98 | Neg | Neg | Neg |
| 4 | 7 | T1-2N0M0 | 6.9 | 60 | Arg/ Arg | 1.59 | Pos | Neg | Pos |
| 5 | 8-10 | T1-2N0M0 | 7.9 | - | Pro/ Arg | 1.98 | Neg | Pos | Neg |
| 6 | 6 | T3-4N0/xM0 | 7.5 | 12 | Pro/ Arg | 1.32 | Pos | Neg | Neg |
| 7 | 7 | T1-2N0M0 | 7.3 | 16 | Arg/ Arg | 1.14 | Neg | Neg | Neg |
| 8 | 6 | T2-4Nx/+M1 | 5.8 | 43 | Arg/ Arg | 1.23 | Neg | Neg | Pos |
| 9 | 7 | T3-4N0/xM0 | 8.2 | 18 | Pro/ Arg | 1.36 | Neg | Neg | Neg |
| 10 | 8-10 | T3-4N0/xM0 | 7.8 | 22 | Pro/ Arg | 1.41 | Neg | Neg | Neg |
| 11 | 7 | T1-2N0M0 | 7.7 | 37 | Pro/ Arg | 1.19 | Pos | Neg | Neg |
| 12 | 7 | T2-4Nx/+M1 | 7.9 | - | Arg/ Arg | 0.99 | Neg | Neg | Neg |
| 13 | 7 | T3-4N0/xM0 | 6.9 | - | Arg/ Arg | 0.98 | Pos | Neg | Neg |
| 14 | 6 | T2-4Nx/+M1 | 6.1 | 18 | Arg/ Arg | 1.47 | Neg | Neg | Neg |
| 15 | 8-10 | T1-2N0M0 | 8.3 | 46 | Pro/ Arg | 1.18 | Neg | Neg | Neg |
| 16 | 7 | T3-4N0/xM0 | 5.2 | 49 | Pro/Pro | 1.21 | Neg | Neg | Pos |
| 17 | 6 | T1-2N0M0 | 5.7 | 20 | Pro/ Arg | 1.23 | Pos | Neg | Pos |
| 18 | 6 | T3-4N0/xM0 | 6.1 | 38 | Arg/ Arg | 1.32 | Neg | Pos | Neg |
| 19 | 7 | T1-2N0M0 | 5.1 | 14 | Pro/ Arg | 1.45 | Neg | Neg | Neg |
| 20 | 7 | T1-2N0M0 | 7.4 | 52 | Arg/ Arg | 1.58 | Neg | Neg | Pos |
| 21 | 8-10 | T2-4Nx/+M1 | 8.1 | - | Pro/ Arg | 0.89 | Neg | Neg | Neg |
| 22 | 6 | T3-4N0/xM0 | 5.8 | 60 | Pro/Pro | 1.26 | Pos | Neg | Neg |
| 23 | 8-10 | T2-4Nx/+M1 | 8.2 | 58 | Pro/ Arg | 1.59 | Neg | Neg | Neg |
| 24 | 7 | T2-4Nx/+M1 | 7.3 | 43 | Arg/ Arg | 1.25 | Neg | Neg | Neg |
| 25 | 8-10 | T1-2N0M0 | 6.9 | 19 | Pro/ Arg | 1.41 | Neg | Neg | Pos |

| | | | | | | | | | |
|----|------|------------|-----|----|---------|------|-----|-----|-----|
| 26 | 7 | T2-4Nx/+M1 | 6.2 | 22 | Pro/Pro | 1.32 | Pos | Neg | Neg |
| 27 | 7 | T1-2N0M0 | 5.2 | 28 | Arg/Arg | 1.15 | Neg | Neg | Pos |
| 28 | 6 | T2-4Nx/+M1 | 5.4 | 57 | Arg/Arg | 1.82 | Pos | Neg | Neg |
| 29 | 6 | T3-4N0/xM0 | 8.0 | 58 | Arg/Arg | 1.34 | Neg | Neg | Neg |
| 30 | 8-10 | T1-2N0M0 | 7.4 | 62 | Pro/Arg | 1.40 | Neg | Neg | Neg |

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